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13. Abstract *(Maximum 200 Words) (abstract should contain no proprietary or confidential information)*

Primary inflammatory breast cancer (IBC) accounts for approximately 6% of new breast cancers annually. We hypothesize that a limited number of concordant genetic alterations give rise to the unique aggressive inflammatory phenotype of IBC. While working on the genetic determinants underlying the IBC phenotype, we found concordant and consistent alterations of two genes, RhoC GTPase and a novel IGF-binding protein (IGF-BP), in patients with IBC. RhoC was overexpressed in 90% of IBC samples examined compared to 30% of stage matched non-IBC tumors. LIBC was lost in 80% of the IBC samples and only 20% of the non-IBC samples examined. Since RhoC and LIBC appear to act in concert in IBC, coupled to the preliminary evidence from other laboratories of genes from these families playing a role in pancreatic cancer (another highly aggressive adenocarcinoma), they are excellent candidate genes to begin to probe the genetic basis of the aggressive phenotype in IBC. We hypothesize that the phenotype of IBC is due to alterations in expression of RhoC and IGF-BP early in tumorigenesis. We will elucidate the signalingpathway downstream from RhoC GTPase and attempt to determine what effect RhoC and LIBC have on cellular motility and invasion.

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Sofia D. Merajver, M.D., Ph.D.
Annual Report for Award Number: DAMD17-00-1-0345
September 12, 2002

Introduction:

This is a progress report for a project that aims at understanding the genetic determinants of Inflammatory Breast Cancer (IBC). In particular, we aim to discern the relative role of the RhoC GTPase gene as well as a gene named WISP3 (formally LIBC) in the specific phenotypic characteristics of Inflammatory Breast Cancer. We have made significant progress in the last year, which is summarized below.

Body:

The major thrusts of our year's work have been: 1) Understanding the functional significance of the WISP3 gene in Inflammatory Breast Cancer (IBC). 2) Testing novel ways to inhibit the effects of RhoC over-expression. 3) Better understanding of RhoC and WISP3 signaling. We have also published a report dealing with the expression of E-Cadherin in Inflammatory Breast Cancer and another paper that for the first time reports that Epstein-Barr virus is associated with multiple breast adenomas in immunocompromised works. Interestingly, our laboratory group was able to define the surprising finding that E-Cadherin expression is preserved as Inflammatory Breast Cancer cells as they enter into the dermal lymphatics; this is not completely unexpected but somewhat of a surprising finding given that, in general, E-Cadherin expression is associated with a lower metastatic potential. Below are brief capsules of key accomplishments:

> WISP3 – This work is seminal, as no other tumor suppressor gene had ever been defined specifically for WISP3.
>
> RhoC – We have had major success in discerning how RhoC signals and how its effects can be suppressed by farnesyl transferase inhibitors (FTI's).
>
> RhoC signaling – We have begun array experiments to understand not just a few, but hopefully, most or all the genes modified by RhoC.

We have completed a study on the effect of farnesyl transferase inhibitors on RhoC manuscript detailing these findings is nearing completion.

In summary, we have had once again a very productive year where we have completed several manuscripts dealing with key aspects of the regulation of RhoC expression in Inflammatory Breast Cancer phenotypes and also the phenotypic effects of RhoC overexpression. In addition, we have completed a major effort in understanding the function of the WISP3 gene as it contributes to the phenotype in Inflammatory Breast

Cancer. Taken together, our work now takes us more fully into devising ways to counteract (i.e. treat) RhoC driven malignancies.

Key Research Accomplishments:

The key research accomplishments are summarized above and the following is a list of new manuscripts and abstracts either published or under review during the last year. We now understand how RhoC signals inside the cell to elicit motility and angiogenesis. We have made significant progress in our understanding of how WISP3 elicits its function in breast cancer. We know that FTI's inhibit RhoC. We discovered that EBV is involved in breast adenomas.

Reportable Outcomes:

We are in a position to definitively state that the angiogenic and motility characteristics of Inflammatory Breast Cancer are to a great extent due to the RhoC GTPase overexpression. In addition, we are able to definitively state that farnesyl transferase inhibitors should have activity against RhoC mediated phenotypes in breast cancer. We can report that RhoC over-expression is an early marker of aggressive breast cancers, even when they are small.

Conclusion:

We are encouraged by our progress. We wish to establish the relationship between WISP3 and ß-catenin in the development of resistance to apoptosis in cancer cells. We want to study all genes influenced by RhoC. These are the directions we are moving on for this year.

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Persistent E-Cadherin Expression in Inflammatory Breast Cancer

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E-cadherin is a transmembrane glycoprotein that mediates epithelial cell-to-cell adhesion. Because loss of E-cadherin expression results in disruption of cellular clusters, it has been postulated that E-cadherin functions as a tumor suppressor protein. The role of E-cadherin in inflammatory breast cancer (IBC), a distinct and highly aggressive form of breast cancer, is largely unknown. The aim of our study was to elucidate whether E-cadherin expression contributes to the development and progression of the IBC phenotype and to investigate any differences in E-cadherin expression between IBC and stage-matched non-IBC. Forty-two breast cancer cases (20 IBC and 22 non-IBC) were identified. Strict and well-accepted criteria were used for the diagnosis of IBC. Clinical and pathologic features were studied, and formalin-fixed, paraffin-embedded tissue sections were immunostained for E-cadherin, estrogen and progesterone receptors (ER and PR, respectively), and HER2/*neu*. Statistical analysis was performed using Fisher's exact test. All IBC uniformly expressed E-cadherin, whereas 15 of the 22 (68%) of the non-IBC expressed the protein ($P = .006$). Intralymphatic tumor emboli in the IBC cases were also all E-cadherin positive. Two IBC tumors demonstrated invasive lobular histology, and both cases were positive for E-cadherin. Of the non-IBC cases, three were invasive lobular carcinomas, and all were positive for E-cadherin. No association was found between E-cadherin expression and ER, PR status, or HER2/neu overexpression. Our study demonstrates that there is a strong association between E-cadherin expression and IBC and suggests that E-cadherin may be involved in the pathogenesis of this form of advanced breast cancer. In our study, we demonstrate that circulating IBC tumor cells strongly express E-cadherin, thereby providing an important exception to the positive association between E-cadherin loss and poor prognosis in breast cancer.

KEY WORDS: Breast cancer, E-cadherin, Inflammatory breast cancer, Metastasis, Tumor emboli.

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Inflammatory breast cancer (IBC) accounts for approximately 6% of new breast cancers in the United States annually. It is the most aggressive and lethal form of locally advanced breast cancer, with a mean 5-year disease-free survival rate of <45% (1–3). IBC has unique clinical and pathological features. Clinically, patients present with skin erythema and nodularity; pathologically, IBC is highly angiogenic and angioinvasive, with numerous tumor emboli filling the dermal lymphatics. These tumor emboli are responsible for the striking clinical picture that arises from lymphatic obstruction (4–6).

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent intercellular adhesion and is specifically involved in epithelial cell-to-cell adhesion (7). Diminished E-cadherin expression has been related to the acquisition of invasiveness in experimental tumors and in advanced-stage carcinomas, including ductal carcinomas of the breast (7–12). Several studies have shown that E-cadherin expression is significantly reduced in high-grade, estrogen receptor (ER)–negative, and metastatic breast carcinomas (10, 13, 14). Loss of E-cadherin expression tends to appear in a heterogeneous pattern within carcinomas, suggesting that loss of expression of E-cadherin in these tumors is a potentially reversible somatic alteration. It is not known to what level circulating malignant cells with metastatic potential express E-cadherin.

A recent study reported overexpression of E-cadherin in IBC both in human and in an IBC xenograft model in SCID/nude mice (15). Given the unique highly metastatic IBC phenotype, we hy-

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pothesized that IBC would exhibit a pattern of E-cadherin expression distinct from stage matched non-IBC. In addition, the high propensity of IBC cells to invade lymphatic channels makes IBC an interesting model to study E-cadherin expression in metastasis-enabled circulating cancer cells.

MATERIALS AND METHODS

Patient Selection

IBC and non-IBC patients were chosen using the computerized clinical database and by prospective identification of newly diagnosed patients. We identified 20 cases of IBC and 22 cases of stage-matched (Stages III and IV) non-IBC. All cases were obtained from the pathology files in our institution, and hematoxylin and eosin–stained slides were available for review in all cases. Strict and well-accepted criteria were used to make the diagnosis of IBC (4–6). Clinically, erythema over at least one third of the breast was required, with the classical peau d'orange appearance that includes skin thickening and erythema, with or without nodularity. Although the diagnosis of IBC is primarily clinical, all the IBC cases also had pathologically demonstrable tumor emboli in the dermal lymphatic channels. The non-IBC tumors presented as either palpable masses or mammographic abnormalities without skin changes, and pathologically, none of these cases contained dermal lymphatic tumor emboli, as assessed in nipple sections of the mastectomy specimens.

Immunohistochemistry

E-cadherin protein expression was studied by immunohistochemistry using specific monoclonal antibodies (Zymed Laboratories, San Francisco, CA). For assessment of ER, progesterone receptor (PR), and HER2/*neu* expression, specific monoclonal antibodies for ER (Ventana Medical Systems, Tucson, AZ), PR (DAKO, Carpinteria, CA), and HER2/neu (Herceptest from DAKO) were used at their manufacturers' recommended dilutions. Immunohistochemistry was performed as previously described using an automated immunostainer (Biotek Techmate 500, Ventana Medical Systems; 16). Briefly, 5-μm-thick sections were cut onto glass slides from formalin-fixed paraffin blocks. Sections were deparaffinized, microwaved (1000-watt Model MTC1080-2A; Frigidaire, Dublin, OH) in a pressure cooker (Nordic Ware, Minneapolis, MN) with 1 L 10 mM citrate buffer, pH 6.0. They were subsequently cooled with the lid on for an additional 10 minutes. After removing the lid, the entire pressure cooker was filled with cold running tap water for 2 to 3 minutes or until the slides were cool. At 36°C, the stainer sequentially added an inhibitor of endogenous peroxidase, the primary antibodies (32 minutes), a biotinylated secondary antibody, an avidin-biotin-complex with horseradish peroxidase, 3,3'-diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride), and copper enhancer. The slides were then counterstained with hematoxylin.

E-cadherin expression was interpreted as either positive or negative. To qualify as positive, complete and crisp membranous staining was required in ≥10% of the tumor cells. In the negative cases, internal positive controls, such as epidermis, lymphocytes, and benign breast lobules, were examined. ER and PR were considered positive when >5% of the tumor cell nuclei were stained. For HER2/*neu*, the strength of the membranous staining was recorded as 0 or as 1+ through 4+, and a sample was considered positive when ≥10% neoplastic cells had a staining intensity of 2+ or greater.

Statistical Analysis

Differences in percentages of E-cadherin positive cases between the IBC and non-IBC groups were tested for statistical significance using Fisher's exact test. A P value of ≤.05 was considered significant. A two-sample t test was also performed to compare the ages at diagnosis of the two groups of patients. Logistic regression was used to examine differences in E-cadherin expression between IBC and non-IBC patients, adjusted for age, ER and PR expression, and HER2/*neu* overexpression.

RESULTS

Clinical and Pathological Features

All patients were female. IBC patients ranged in age at diagnosis from 35 to 72 years (mean age, 51), and non-IBC patients ranged in age from 31 to 78 years (mean age, 591). Twenty cases were diagnosed as IBC, and 22 cases as non-IBC. Thirty-four tumors (81%) were invasive ductal carcinomas (18 IBC, 16 non-IBC), five tumors (12%) were invasive lobular carcinomas (2 IBC, 3 non-IBC), one tumor was a medullary carcinoma (non-IBC), and another was an atypical medullary carcinoma (non-IBC). One tumor was an anaplastic carcinoma (non-IBC). There were no statistically significant differences in the frequency distribution of histologic tumor types between IBC and non-IBC tumors.

E-Cadherin Expression in IBC and Non-IBC Tumors and Its Relationship to ER, PR, HER2/*neu* Expression, and Angiolymphatic Invasion

All IBC strongly expressed E-cadherin, characterized by crisp staining of cell membranes in ≥10% of

the cells with a staining intensity of 2+ or more. Of note, the endolymphatic tumor emboli were also strongly positive for E-cadherin in all cases (Fig. 1). Of the 22 non-IBC tumors, 15 (68%) expressed E-cadherin and 7 (32%) cases did not. Table 1 shows the frequency of E-cadherin expression in IBC *versus* non-IBC. The difference in E-cadherin expression rates in IBC *versus* non-IBC was statistically significant ($P < .006$; Fig. 2). To exclude the possibility that the significant difference in E-cadherin expression between IBC and non-IBC tumors was influenced by age, ER, PR, and HER2/

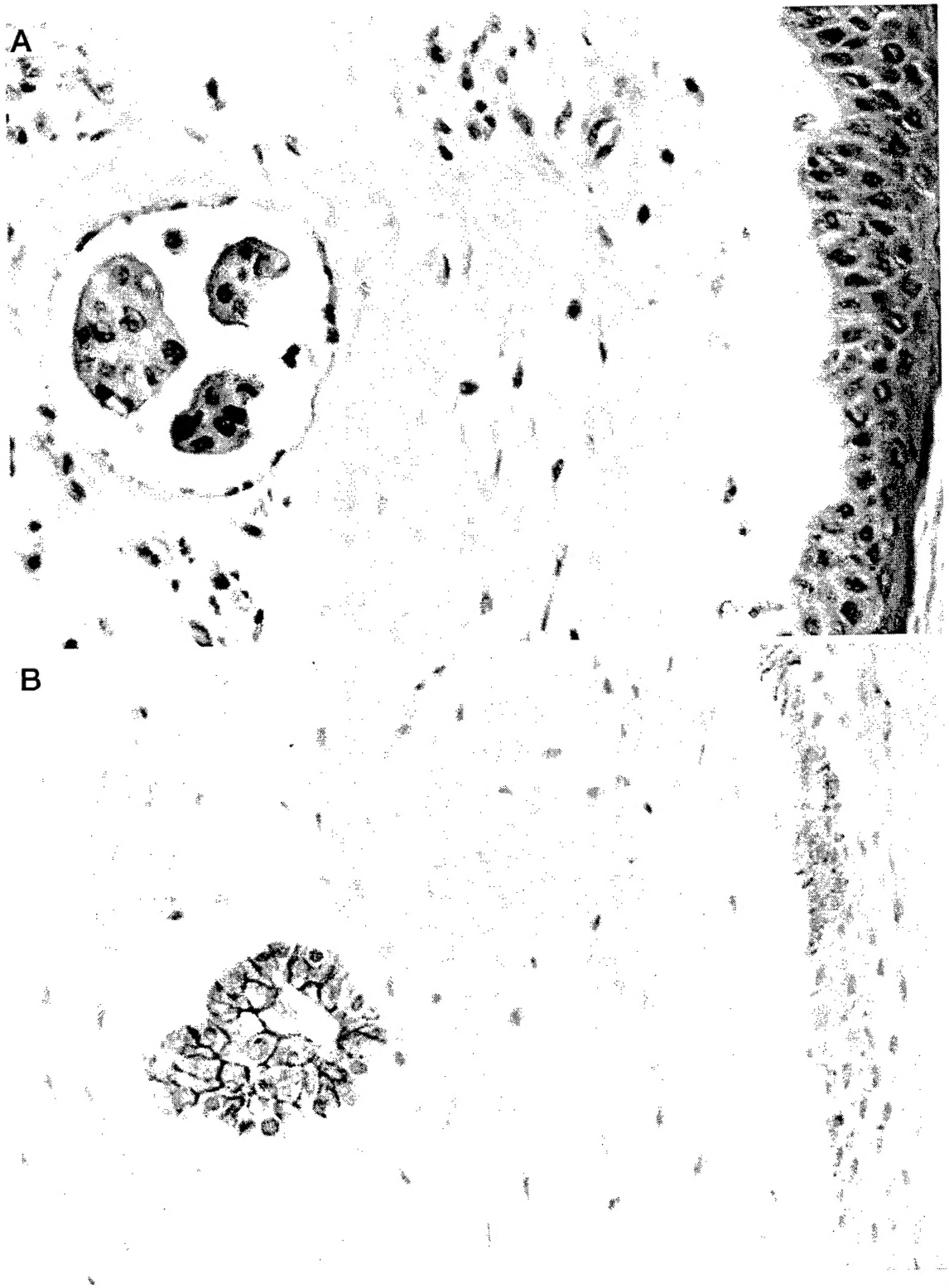

FIGURE 1. Inflammatory breast cancer with a characteristic tumor embolus in a dermal lymphatic channel (**A**) and strong and crisp membranous staining for E-cadherin in the intralymphatic tumor cells (**B**). Magnification: ×20.

TABLE 1. Relationship between E-Cadherin Expression, ER, PR, and HER2/*neu* Status in IBC *versus* Non-IBC Patients

	IBC: # of Positive Cases (%)	Non-IBC: # of Positive Cases (%)	*P* Value
E-cadherin expression	20 (100)	15 (68)	=.006
ER expression	7 (41)	8 (38)	>.05
PR expression	6 (35)	9 (43)	>.05
HER2/*neu* overexpression	5 (56)	6 (43)	>.05

ER, estrogen receptor; PR, progesterone receptors; IBC, inflammatory breast cancer; non-IBC, non-inflammatory breast cancer.

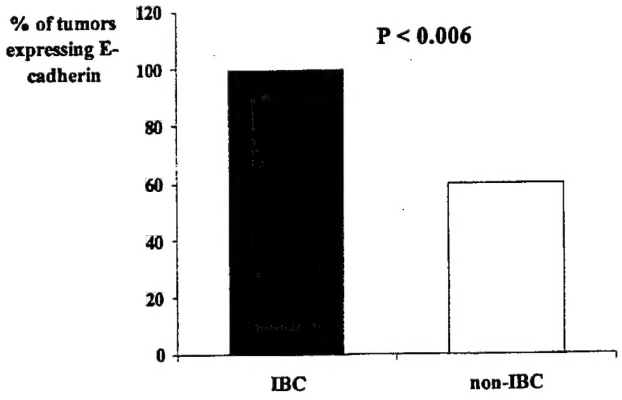

FIGURE 2. Analysis of E-cadherin expression in inflammatory breast cancer (IBC) *versus* non-IBC. The bars illustrate the statistically significant difference in E-cadherin expression between the two groups of tumors.

neu status, we used logistic regression after adjusting for these variables. The increased expression of E-cadherin in patients with IBC, as compared with non-IBC patients, remained statistically significant after adjustment. Interestingly, the two IBC with features of invasive lobular carcinomas expressed E-cadherin, in contrast to the three cases of invasive lobular carcinoma in the non-IBC group, which were all negative for E-cadherin (Fig. 3).

Of the 22 stage-matched, non-IBC tumors, 17 (77%) had angiolymphatic invasion and/or lymph node or distant metastases, and 5 tumors (23%) did not. E-cadherin was expressed in 11 (65%) and in 4 (80%) of the non-IBC with and without angiolymphatic invasion and/or metastases, respectively ($P < .005$). Thus, we observed a significant relationship between loss of E-cadherin expression and angiolymphatic invasion for non-IBC tumors.

Among the IBC tumors, seven cases (41%) were ER positive, and six cases (35%) were PR positive, equivalent to the non-IBC distribution, in which eight tumors (38%) expressed ER and nine tumors (43%) expressed PR. HER2/neu was overexpressed in five (56%) IBC tumors and in six (43%) of non-IBC tumors. Table 1 shows the relationship between E-cadherin expression, ER/PR status, and HER2/*neu* overexpression. No statistically significant differences in ER, PR, or HER2/*neu* status were observed between IBC and non-IBC tumors.

DISCUSSION

IBC is a very distinct form of breast carcinoma with unique clinical and pathologic features that pursues an extremely aggressive course (3, 4, 6). Because of these unique characteristics, we hypothesized that distinct genetic alterations may define the inflammatory phenotype. The present study provides a new insight into the E-cadherin–mediated cell-to-cell adhesion in the pathogenesis and/or progression of IBC as it demonstrates that E-cadherin is expressed in 100% of IBC and is preferentially expressed in IBC when compared to stage matched non-IBC.

The results of our study agree with a recent report by Alpaugh *et al.* (15), who developed a human xenograft model of IBC in SCID/nude mice that closely recapitulates the pathology of IBC in humans. These investigators detected by Western blot analysis 10- to 20-fold overexpression of E-cadherin in the xenografts and confirmed E-cadherin overexpression by immunohistochemistry in the xenografts and in cases of human IBC.

Compelling evidence exists in the literature to indicate that down-regulation of E-cadherin expression and/or function is a critical factor in the malignant progression of epithelial tumors (8, 11, 12). Transfection of E-cadherin into invasive carcinoma cell lines reduced their ability to invade *in vitro*, further supporting the role of E-cadherin in maintaining cells in an epithelial ordered state and suppressing the invasive potential of nascent malignant cells (11, 12). Furthermore, restoration of E-cadherin expression, initially lost in the transition from adenoma to invasive carcinoma, resulted in tumor arrest at the adenoma stage in a transgenic mouse model of pancreatic B-cell carcinogenesis (8). E-cadherin is thought to act as a tumor suppressor gene in the breast, although the mechanism of E-cadherin–mediated tumor suppression has not been fully elucidated (17). Previous reports showed low expression of E-cadherin in breast cancers with increased invasiveness and high metastatic potential (13, 14). Our results are in agreement with the literature in the sense that the non-IBC tumors with angiolymphatic invasion and/or metastases had significantly less E-cadherin expression than the tumors that did not have these features. Most important, we demonstrate that in IBC, the most aggressive form of breast cancer, 100% of cases show E-cadherin protein expression, regardless of the histologic type of the tumor or of ER, PR, or HER2/*neu* expression. It is very likely that previous studies comprised highly heterogeneous groups of tumors

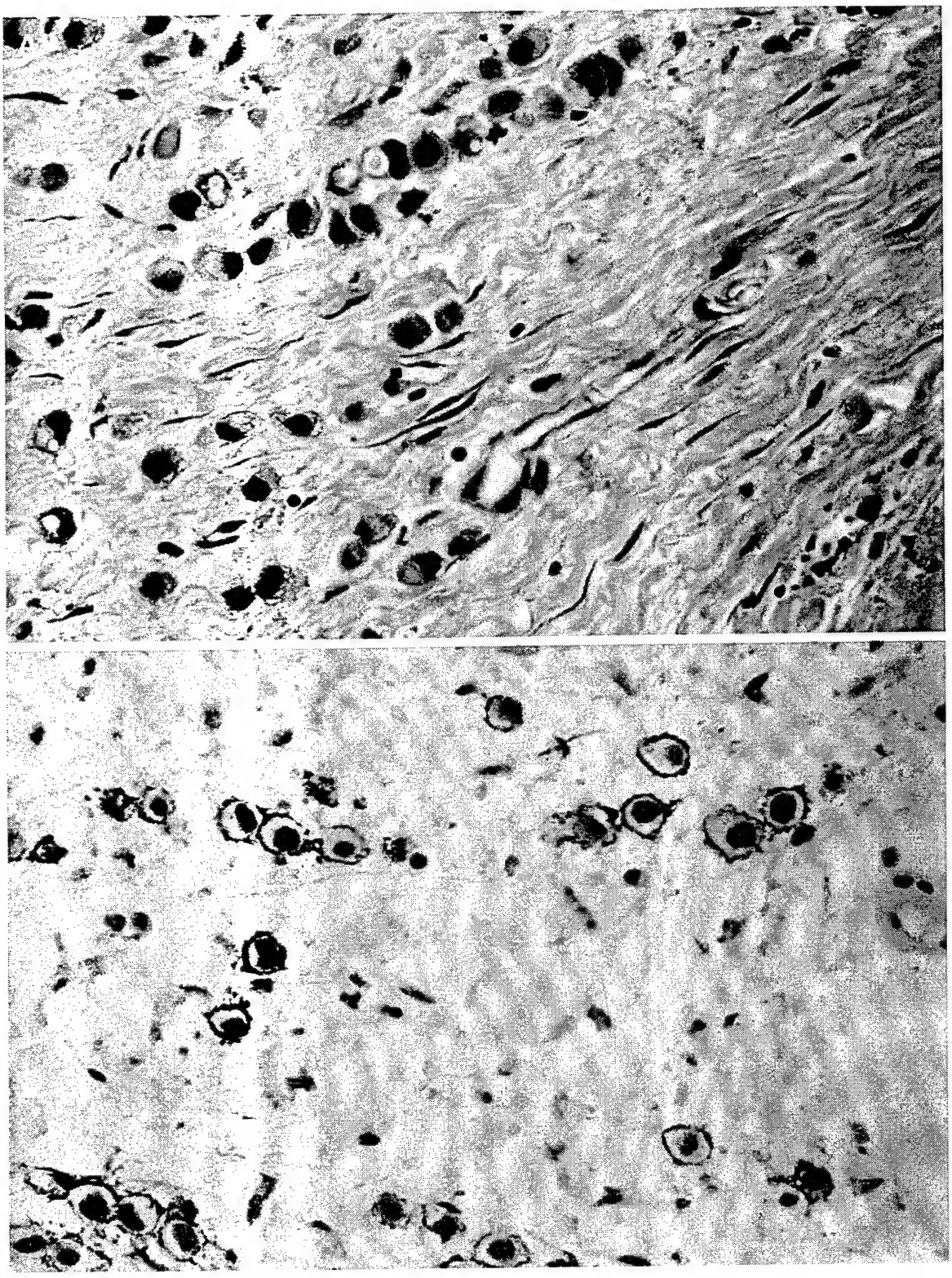

FIGURE 3. Primary inflammatory breast cancer with classic invasive lobular carcinoma histology, composed of files of small malignant cells, some of which have signet-ring cell features (**A**). Positive E-cadherin staining in the malignant cells with classic lobular morphology (**B**). The membranous staining highlights the signet-ring cell features of some of the neoplastic cells. Magnification: ×40.

in which very few cases would have been IBC. Our results indicate that E-cadherin does not appear to function as a tumor suppressor gene in IBC, and they are in support of recent studies that suggest a role for E-cadherin in cellular differentiation and survival (18–21). The different potential roles of E-cadherin in the pathogenesis of IBC and non-IBC warrant further investigation.

To metastasize, cancer cells must break away from the primary tumor, move into the surrounding

stroma, intravasate into the lymphatic or vascular circulation, extravasate, and successfully reestablish growth at other sites. Although it has long been postulated that in the course of metastasis development cancer cells lose E-cadherin expression and thereby intercellular adhesion, *in vivo* and *in vitro* studies have failed to correlate reduced E-cadherin expression with an invasive and metastatic phenotype (10, 22). Moreover, it is unknown whether E-cadherin expression is reduced in circulating cells before extravasation. In the present study, intralymphatic tumor cells strongly express E-cadherin, which challenges the hypothesis that loss of expression is a necessary event in the circulating cancer cells as they become metastasis enabled. On the basis of our results and the results of other investigations (23, 24), we suggest that loss of E-cadherin expression is a transient phenomenon that has the purpose of allowing malignant cells to invade vascular channels and tissues; we further suggest that once in the circulation, these cancer cells reinstate the expression of E-cadherin, facilitating intercellular adhesion and enabling the formation of cohesive tumor emboli. Interestingly, when analyzing E-cadherin expression at the primary site of non-IBC tumors, we found a highly significant correlation between loss of E-cadherin expression and presence of angiolymphatic invasion. This is consistent with other observations indicating that in non-IBC, loss of E-cadherin is a poor prognostic marker.

In breast cancer, reduced expression of E-cadherin has been reported in approximately 50% of invasive ductal carcinomas, whereas invasive lobular carcinomas showed complete loss of E-cadherin expression in nearly 90% of cases (17, 24, 25). Truncating E-cadherin mutations have been found in two thirds of invasive lobular carcinomas but in no invasive ductal carcinomas (26, 27). These studies suggest a relationship between loss of E-cadherin–mediated cell adhesion and the diffuse and discohesive pattern of growth that is characteristic of invasive lobular carcinoma. In the present study, two E-cadherin–positive IBC were histologically classic invasive lobular carcinomas. Of the non-IBC, three cases were invasive lobular carcinomas, all of which were E-cadherin negative. Despite the fact that the small number of cases precludes drawing firm conclusions on the possible relationship between the IBC phenotype, invasive lobular carcinoma histology, and E-cadherin expression, our results suggest that E-cadherin expression may have an even stronger positive association with the IBC phenotype than loss of E-cadherin expression does with the lobular morphology.

In conclusion, our study demonstrates a strong positive association between E-cadherin expression and the IBC phenotype. We further demonstrate that circulating tumor cells of IBC patients strongly express E-cadherin and that thus, IBC constitutes an important exception to the association between loss of E-cadherin expression and increased metastatic potential and poor outcome in breast cancer.

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Risk of Breast and Ovarian Cancer in Women With Strong Family Histories

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ABSTRACT

Assessing the risk of breast and ovarian cancer starts with obtaining a complete and accurate family history. This can reveal evidence of inherited cancer risk. The highest risk of cancer is associated with germ-line abnormalities in several genes, including BRCA1, BRCA2, and TP53. Moderate-risk genes associated with syndromes that are inherited in an autosomal dominant pattern (such as Cowden's disease, hereditary nonpolyposis colorectal cancer, Muir-Torre syndrome, and Peutz-Jeghers syndrome) exhibit lower penetrance and thus less risk of breast and/or ovarian cancer. Low-risk genes likely require significant environmental exposure, and although they are associated with the lowest risk of cancer, they account for more cancer than high- and moderate-risk genes. Lifetime risks for breast or ovarian cancer can be estimated. The Gail and Claus models, the more widely utilized models for calculation of lifetime breast cancer risk, are discussed. Models are also available for determining the likelihood of finding a BRCA1/2 mutation (the BRCAPRO and Myriad models). Appropriate candidates for testing include affected individuals who are most likely to have a hereditary form of cancer. Testing should proceed only after a thorough discussion of the risks, benefits, and limitations of testing. Risk-reducing options are available to women with a strong family history of breast and ovarian cancer. These options include high-risk screening, chemoprevention, and prophylactic surgery.

Approximately 30% of individuals with breast cancer have some family history of cancer (generally breast and/or ovarian cancer).[1,2] However, the majority of women who present with a family history of cancer will not have a history that is suggestive of an autosomal dominantly inherited disorder (cancer in multiple generations). It is estimated that only 5% to 10% of breast and ovarian cancers are associated with a strong family history of cancer and are, therefore, likely to be attributed to inheritance of a mutation in a known cancer-causing gene.[3] Current information suggests that these genes may be grouped into high-, moderate-, and low-risk genes.[4] Genes that confer the greatest lifetime cancer risk can be thought of as high-risk genes.

In this article, we will focus on BRCA1, BRCA2, and TP53 (aka p53) as high-risk genes.[3] Moderate-risk genes are inherited in an autosomal dominant fashion with lower penetrance and therefore less cancer risk. Low-risk genes can be associated with the smallest cancer risk. These genes and their risk for cancer are less well understood, and the associated cancer risks are controversial. Likely, cancer attributable to low-risk genes develops later in life and is linked to significant environmental exposure.[5,6]

The commercial availability of genetic testing for cancer risk introduces a new dimension in the assessment of breast and ovarian cancer risk. Genetic testing for cancer risk is a complex process. Appropriate individuals for testing should have the highest likelihood, within a family, of carrying a cancer-causing gene mutation. Before undergoing genetic testing, individu-

Table 1

Features of Hereditary Breast and Ovarian Cancer

- Early onset (diagnosed < age 50) or bilateral breast cancer
- Breast and ovarian cancer occurring in the same individual
- Presence of personal or family history of male breast cancer
- Ashkenazi Jewish ancestry in association with family history of breast or ovarian cancer
- Multiple cases of early breast cancer (diagnosed < age 50) and ovarian cancer at any age
- Other cancers that may indicate the presence of a hereditary cancer syndrome: brain, adrenocortical cancers, sarcoma, leukemia, nonmedullary thyroid carcinoma, colon cancer, endometrial cancer, pancreatic cancer

als must understand the risks, benefits, and limitations of genetic testing. This includes understanding the psychological and ethical issues that surround genetic testing.

The first steps in this process are gathering an accurate family history and recognizing the features of hereditary breast and ovarian cancer. It is also important to understand the options that are available for individuals who are at high risk of cancer (those with a family history or found to be gene mutation carriers).

High-risk cancer screening is available; however, these screening recommendations are based on expert opinions and are largely unproven. Options for cancer prevention include prophylactic surgery (oophorectomies, mastectomy) and chemoprevention. The ability to counsel and provide an accurate assessment of risk and appropriate screening recommendations for these individuals is critical for the provider. We present guidelines for risk assessment in individuals with a family history of breast and ovarian cancer and review the management issues involved in caring for this group of women.

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Gathering the History

Even in this era of microchip technology, an accurate family history continues to be the most informative tool for assessing cancer risk. Despite its importance in assessing risk, obtaining a family history is rapidly becoming a lost skill. Significant deficits exist in the documentation of family history in the primary care setting. This may stem from a diminution of the time that is available for physician and patient interactions, as well as a lack of emphasis during physician training. In one cross-sectional study, a family history was obtained in only 50% of new patient visits and was updated in 22% of established patient visits.[7]

The first step in obtaining an accurate cancer family history involves gathering information to generate a pedigree that spans at least three generations. The history should include all types of cancer in both the paternal and maternal lineage, since breast and ovarian cancer risks can be transmitted through the father. For each affected relative, it is important to try to ascertain the site of the primary tumor, laterality of disease, age at diagnosis, treatment, and age at death. Eliciting information about unaffected family members is key to interpreting the family history and should include cause of and age at death. If the total number of female relatives is small, significant expression of an inherited gene may not be apparent. Gathering information about the presence of other chronic diseases in the family, such as osteoporosis and coronary artery disease, is important since this information may influence management recommendations (eg, recommendations for hormone replacement therapy).

Studies have shown that family history information that identifies breast or colon cancer as a primary cancer site in first-degree relatives may be accurate in 89% to 91% of cases.[8] However, the accuracy rate for such information decreases in the case of second- and third-degree relatives. Often, the diagnosis of premalignant conditions may be confused with cancer, and sites of recurrence can be misidentified as second primary tumors. Hence, procuring pathology reports or medical records to verify a family history of cancer is strongly recommended. Ascertaining ancestry is an integral component of the process. The existence of specific mutations (eg, BRCA1/BRCA2) in certain populations makes ethnicity-based testing an important option to consider.

Current advances in technology may incorporate new tools such as computer programs to record genetic information,[9] but physicians will continue to play an important role in the interpretation of this information and the definition of risk. Future developments in genetic research will only serve to increase the importance of an accurate family history. All physicians should develop methods to capture family history at the time of an initial patient visit and then periodically update this information.

Features of Hereditary Breast and Ovarian Cancer

Certain features of an individual's personal or family history may lead the clinician to suspect an inherited predisposition to breast and/or ovarian cancer. A personal history of early breast cancer (onset at age < 50 years) or bilateral breast cancer may be a clue to a genetic predisposition. Additionally, breast and ovarian cancer in the same individual may herald the presence of hereditary malignancies. As we will discuss later, a history of ovarian cancer or early breast cancer plus Ashkenazi Jewish ancestry carries a significant risk of an inherited form of the diseases.

Inherited factors may play a role when the above personal history coexists with early-onset breast cancer, bilateral breast cancer, male breast cancer, or ovarian cancer in more than one generation. The presence of other cancers in the family, such as cancers occurring at early ages (eg, colon cancer at age < 50 years) or cancers without the usual risk factors (such as lung cancer in a nonsmoker) may be clues to an inherited form of cancer. See Table 1 for a list of other cancers that

can be associated with hereditary breast or ovarian cancer.

Families vary in size, and it is important to take into account the number of individuals within a family, as well as the number of women at risk. For example, a small family with few women over age 40 diagnosed with one early breast cancer and ovarian cancer can be significant, whereas a very large family with several women over age 60 affected with breast cancer may be less likely to carry a genetic alteration in BRCA1/2.

Breast and ovarian cancer are features of several hereditary syndromes. Families with clear autosomal dominant patterns of inheritance are in the minority (5% to 10% of all individuals with either breast or ovarian cancer).[3] More than 70% percent of such families will be found to carry mutations in BRCA1 or BRCA2.[3] Individuals with BRCA mutations have high lifetime cancer risks and will be discussed below as high-risk genes. Other syndromes, such as Cowden's disease, hereditary nonpolyposis colorectal cancer (HNPCC), Muir-Torre syndrome, and Peutz-Jeghers syndrome also display autosomal dominant patterns of inheritance. However, these carry a lower penetrance and, therefore, a lesser lifetime risk of cancer. These syndromes have a moderate lifetime cancer risk, and the genes associated with the syndromes have often been termed moderate-risk genes.

Low-risk genes carry the lowest risk of cancer (relative risks of 2.0 to 7.0) but are likely to be more prevalent than either high- or moderate-risk genes. Therefore, they contribute more to the overall development of cancer. To date, low-risk genes have not been associated with ovarian cancer, and consequently, only low-risk genes associated with the risk of breast cancer will be discussed in this article. See Table 2 for a list of high-, moderate-, and low-risk genes, their associated syndromes, and lifetime risk of breast and/or ovarian cancer.

High-Risk Genes

The greatest lifetime risk of breast and ovarian cancer is conferred by mutations in either BRCA1 or BRCA2. Since their identification, we have learned much about these two genes over the years. First identified in 1994,[10] BRCA1 is a large gene located on chromosome 17. The gene codes for a protein, 1,836 amino acids in length. More than 500 mutations have been detected in this gene. BRCA2, identified in 1995, is approximately twice as large as BRCA1 and is located along chromosome 13.[11] Over 300 mutations in this gene have been detected. The exact functions of BRCA1/2 are unclear; however, evidence suggests that these genes are involved in DNA repair.[12]

Mutations in BRCA1/2 account for a small percentage of all breast or ovarian cancer cases. However, they account for the majority (70%) of hereditary breast and ovarian cancers, with "other" genes held responsible for the remaining 30%.[3,13] Much research has been carried out to quantitate the contribution of hereditary breast and/or ovarian cancer to specific populations.[14] Several studies have examined groups of young women with breast cancer.[15-18] In aggregate, these studies suggest that the contribution of BRCA1 to early-onset breast cancer (diagnosed before age 45) is less than 10%. In all of these studies, a woman with early breast cancer was more likely to carry a mutation if she had a family history of breast cancer.

Founder mutations are genetic abnormalities that are commonly seen in a specific population. They are found when a population has been reduced to small numbers because of war, famine, or geographic isolation, and then expands. The most clinically important examples of this phenomenon are the three founder mutations (two in BRCA1 and one in BRCA2) that exist in the Ashkenazi Jewish population.[19,20] Studies of Jewish women suggest that approximately 30% of Jewish women diagnosed with breast cancer prior to age 45, and 30% to 60% of Jewish women diagnosed with ovarian cancer will harbor one of the three common mutations.[21-23] These three mutations account for more than 90% of all BRCA mutations in the Jewish population.

Women with mutations in BRCA1 or BRCA2 have a 56% to 87% probability of developing breast cancer by age 70.[24,25] The risk of a second primary cancer (often defined as breast cancer in the contralateral breast or separate quadrant of the ipsilateral breast) is thought to be between 40% and 60%.[26]

Ovarian cancer risk differs between these two genes. BRCA1 is associated with a 15% to 44% lifetime risk of ovarian cancer, while BRCA2 has a 10% to 27% lifetime risk.[26,27] There is a risk of prostate and colon cancer for carriers of BRCA1 mutations, with relative risks of 3.3 and 4.0, respectively.[25] Men with mutations in BRCA2 have a 6% lifetime risk of male breast cancer.[26] Individuals with BRCA2 mutations appear to be at risk for other types of cancer as well.

Cancers with an estimated relative risk greater than 2.0 include gastric, gallbladder, pancreatic, and prostate carcinoma, as well as melanoma.[26] The probability of finding a mutation in BRCA1/2 based on a specific family history is discussed later. Further information regarding BRCA1/2 can be found in the Breast Cancer Linkage Consortium website (ruly70.medfac.leidenuniv.nl).

Li-Fraumeni syndrome is a cancer family syndrome associated with multiple types of cancer (soft-tissue sarcomas, breast carcinoma, brain tumors, leukemia, and adrenal cortical carcinoma), with cancer often presenting at very early ages. Germ-line mutations in TP53, a tumor-suppressor gene, have been identified in these families.[28] Breast cancer is a significant component of this syndrome, with the majority of patients being affected before age 45, and with a high incidence of bilateral metachronous tumors.[29] The lifetime risk of cancer may be higher than 50%; however, there are no published studies that quantify breast cancer risk with this syndrome.

Moderate- and Low-Risk Genes

Compared to BRCA1 and BRCA2, the role of moderate- and low-risk genes in the causation of breast and ovarian cancer is less well defined. As we have discussed above, moderate-risk genes have lower penetrance than BRCA1/2 (conferring a lower lifetime cancer risk). Low-risk genes include those genes associated with chromosome fragility, carcinogen metabolism, and regulation of steroid hormone levels. Little information is available regarding ovarian cancer and low-risk genes, and therefore, our discussion will focus only on breast cancer risk.

Table 2

Genetic Syndromes and Lifetime Cancer Risks

Genes	Syndrome/Gene Function	Breast Cancer	Ovarian Cancer	References
		Lifetime Risk		
High Risk				
BRCA1	Hereditary breast/ovarian cancer	56%–87%	15%–44%	[24,25,27]
BRCA2	Hereditary breast/ovarian cancer	56%–87%	10%–27%	[24,26]
TP53	Li-Fraumeni syndrome	NQ	NQ	
Moderate Risk				
MSH2/MLH1	Hereditary nonpolyposis colorectal cancer	NQ	9%	[34]
MSH2/MLH1	Muir-Torre syndrome	NQ	N/A	[38]
PTEN	Cowden's syndrome	30%–50%	N/A	[32]
STK11	Peutz-Jeghers syndrome	NQ	N/A	[34]
		Relative Risk		
Low Risk				
ATM	Ataxia telangiectasia	1–6.8	N/A	[39,40]
CYP1A1	Carcinogen/hormone metabolism	1–5.65	N/A	[45,46]
CYP17	Carcinogen/hormone metabolism	1–2.5	N/A	[47,48]
GSTM1	Carcinogen metabolism	1–2.1	N/A	[43-45]
NAT2	Carcinogen metabolism	1–4.4	N/A	[41,42]
COMT	Estrogen metabolism	1–2.1	N/A	[49,50]
HRAS1	Proto-oncogene	2.0	N/A	[51]

N/A = no elevated lifetime risk for that particular cancer; NQ = elevated risk for cancer but lifetime risk has not been quantitated.

Low-risk genes will be far less penetrant than moderate- and high-risk genes, but may actually account for more cases of breast cancer because alterations in these genes are likely to be more prevalent. Clinically, the tumors attributable to low-risk genes are likely to present at a later age, and the usual pattern of cancer in every generation may not be seen.[4] See Table 2 for lifetime cancer risks associated with the genes discussed below.

• **Moderate-Risk Genes**—Syndromes associated with moderate risk of breast and/or ovarian cancer likely account for a very small proportion of hereditary breast and ovarian cancer. Cowden's disease is a rare disorder that is inherited as an autosomal dominant trait. Individuals often have pathognomonic facial trichilemmomas, oral mucosal papillomas, and fibroadenomas of multiple organs (breast and uterine being the most common). Breast cancer has been described in a large number of female patients with this syndrome.[30] A lifetime risk estimate of 30% to 50% for breast cancer has been quoted for patients with this syndrome.[31] Germ-line mutations of PTEN have been associated with Cowden's disease kindreds.[32] Individuals with either Cowden's disease or germ-line mutations of PTEN may also be at risk of nonmedullary thyroid carcinoma.

Peutz-Jeghers syndrome is an autosomal dominant disorder characterized by mucocutaneous hyperpigmentation, hamartomatous polyps, and malignancy. Germ-line mutations in STK11, a gene coding for a serine/threonine kinase, have been found in families with Peutz-Jeghers syndrome.[33] The incidence of breast cancer associated with this syndrome is not well characterized. Women in these families tend to develop early and bilateral breast cancer.[34]

Hereditary nonpolyposis colorectal cancer is an autosomal dominantly inherited syndrome associated with an increased risk of ovarian cancer, as well as other cancers. Germ-line mutations in the family of DNA mismatch repair genes (MSH2, MLH1, MSH6, PMS1, and PMS2) can be found in HNPCC families. Members of HNPCC kindreds have a high lifetime risk for both colonic and extracolonic cancers. An analysis of the Finnish HNPCC Registry data revealed a lifetime risk of 9% for ovarian cancer among putative gene carriers.[35] Although molecular evidence suggests that breast cancer may be associated with the HNPCC syndrome,[36] the frequency and lifetime risk are unknown.

A syndrome similar to HNPCC, Muir-Torre syndrome is defined by a detected association between malignancy (colonic and extracolonic) and sebaceous skin tumors (sebaceous gland tumors and keratoacanthomas). The spectrum of malignancies seen in these patients is similar to HNPCC, and germ-line mutations of MSH2 and MLH1 have been identified in individuals with Muir-Torre syndrome.[37] Both benign and malignant breast tumors are seen in patients with Muir-Torre syndrome; however, a clear lifetime risk of breast cancer is not known.[38]

• **Low-Risk Genes**—Ataxia telangiectasia (AT) is an autosomal recessive disease associated with mutations in the ATM gene, an elevated lifetime risk of leukemia and lymphomas, and sensitivity to radiation-induced damage. The role of the ATM gene in breast cancer risk is controversial. Studies[39] have demonstrated an elevated risk of breast cancer for AT heterozygotes. However, subsequent studies have provided conflicting data.[40] Increased utilization of mammographic

Table 3

Lifetime Risk of Breast Cancer Based on Family History of Breast Cancer: The Claus Model

	Age (yr) of Onset of Cancer in Relative					
	20–29	30–39	40–49	50–59	60–69	70–79
Risk With One Affected First-Degree Relative	.211	.165	.132	.110	.096	.088
Risk With One Affected Second-Degree Relative	.142	.120	.104	.094	.094	.083

Risk With Two Affected First-Degree Relatives

Age (yr) of Onset of Cancer in First Affected Relative	Age (yr) of Onset of Cancer in Second Affected Relative					
	20–29	30–39	40–49	50–59	60–69	70–79
20–29	.484	.460	.434	.397	.354	.308
30–39	.460	.437	.399	.353	.302	.252
40–49	.434	.399	.354	.300	.246	.200
50–59	.397	.353	.300	.245	.195	.158
60–69	.354	.302	.246	.195	.156	.128
70–79	.308	.252	.200	.158	.128	.109

Risk With Two Affected Second-Degree Relatives

Age (yr) of Onset of Cancer in First Affected Relative	Age (yr) of Onset of Cancer in Second Affected Relative					
	20–29	30–39	40–49	50–59	60–69	70–79
20–29	.262	.256	.245	.231	.211	.189
30–39	.256	.245	.230	.200	.186	.162
40–49	.245	.230	.209	.184	.159	.137
50–59	.231	.200	.184	.158	.135	.117
60–69	.211	.186	.159	.135	.116	.103
70–79	.189	.162	.137	.117	.103	.094

Risk With One Affected First- and Second-Degree Relative

Age (yr) of Onset of Cancer in First-Degree Relative	Age (yr) of Onset of Cancer in Second-Degree Relative					
	20–29	30–39	40–49	50–59	60–69	70–79
20–29	.450	.433	.407	.369	.320	.264
30–39	.437	.414	.377	.329	.274	.219
40–49	.417	.383	.338	.281	.225	.177
50–59	.388	.343	.289	.233	.182	.143
60–69	.349	.296	.239	.188	.148	.120
70–79	.305	.248	.196	.154	.124	.105

Adapted from Claus et al.[59]

screening by individuals who have one or more abnormal copy of the ATM gene may be associated with an increased risk of breast cancers. However, no studies to date have been able to demonstrate this.

Polymorphisms are genetic abnormalities that are frequently seen in the general population. They have little or no significant impact on the structure or function of the gene. Specific polymorphisms in genes that code for a number of enzymes involved in carcinogen metabolism have been associated with increased risk of breast cancer. The studies in this field often reveal conflicting results. In Table 2, we have attempted to outline cancer risk from the best-studied genes (CYP1A1, CYP17, GSTM1, and NAT2).[41-48]

Hormone exposure is believed to be a significant risk factor for the development of breast cancer. Catechol-O-methyltransferase (COMT) catalyzes the O-methylation of catechol estrogens, which causes inactivation. Low-activity alleles of COMT were reported to be associated with increased breast cancer risk in postmenopausal women with a high body mass index (odds ratio [OR] = 3.58).[49-50] Additional studies have not been able to support these findings.

The HRAS1 proto-oncogene has function in mitogenic signal transduction. It is tightly linked to a microsatellite locus downstream. The microsatellite region is highly polymorphic and certain mutant alleles are associated with an increased risk of breast (OR = 2.29) and other cancers.[51]

The current level of information on low-risk genes is inadequate to move testing into the clinical setting. Genet-

ic polymorphisms, however, are more prevalent than high-risk genes and may ultimately account for a higher population-attributable risk.[6]

Assessing Personal Risk of Breast or Ovarian Cancer

It is well known that women often overestimate their personal risk of breast cancer.[52-53] For women who have breast and ovarian cancers in their families, providing an accurate assessment of lifetime cancer risk can reduce cancer-related anxiety. This is often an important function of cancer-risk assessment clinics. Today, every woman has a 12.2% lifetime risk of breast cancer and a 1.5% lifetime risk of ovarian cancer.[54-55] Although the exact cause of either malignancy remains unknown, risk factors for these diseases do exist.

The two most important risk factors for either breast or ovarian cancer are age and family history. Having one first- or second-degree relative with ovarian cancer increases the lifetime risk of ovarian cancer to between 5% and 7%.[56] To date, there are no more accurate estimates for the lifetime risk of ovarian cancer based on family history or other risk factors.

Having a first- or second-degree relative with breast cancer can significantly increase a woman's lifetime risk of breast cancer.[57] Several models exist for the assessment of lifetime risk of breast cancer: The Gail and Claus models are the most commonly utilized examples.[58,59]

The Gail Model

The Gail Model combines a variety of hormonal and other risk factors (onset of menarche, age at first live birth, number and outcome of breast biopsies) with a very limited family history (number of first-degree relatives with breast cancer).

The Gail Model has been validated and proven to accurately predict risk of breast cancer.[60] This model is quite limited in evaluating potential hereditary breast cancer. The family history is limited to first-degree relatives, thus excluding paternal transmission of cancer. Additionally, no information about other types of cancer, age of cancer diagnoses, or ethnicity (other than white, black, Asian, and Hispanic) is obtained. Calculation of 5-year and lifetime breast cancer risks according to the Gail Model can be performed by accessing the National Cancer Institute's website (www.nci.nih.gov) and searching for information on breast cancer risk.

The Claus Model

The Claus model provides more helpful information for women who have a family history of breast cancer.[59] Using the first- and second-degree family history and age at diagnosis, a lifetime risk of breast cancer can be derived. Thus, a woman with a mother and sister diagnosed with breast cancer, both at age 45, would have a 35% lifetime risk of developing breast cancer (see Table 3). This model is most effective when there are only one or two relatives with cancer. Thus, it may underestimate risk when there is a high likelihood of a BRCA1/2 mutation. Additionally, this model does not take into account cases of ovarian cancer and may underestimate cancer risk where there is paternal transmission of cancer.

Assessing the Probability of BRCA1/2 Mutations

The clinical availability of genetic testing of cancer risk means that we now have ways to more clearly quantitate cancer risk for individuals with a strong family history of cancer. If there is a known genetic alteration within a family, then specific individuals may undergo genetic testing and find that they are either at the general population risk of cancer (no mutation identified) or at a greatly increased risk of cancer (mutation-positive). It becomes important to identify which families are the best candidates for genetic testing. The first step in this process is to gather a complete family history, as outlined above.

Quantifying the probability of finding a mutation in the two most common breast cancer genes, BRCA1 and BRCA2, is difficult. For other hereditary cancer syndromes, criteria have been established to assist in identifying individuals who are most likely to have inherited cancer. Clinical criteria exist for identifying Li-Fraumeni syndrome families, and Amsterdam or Bethesda criteria are used for identifying families that are likely to have HNPCC.[29,61,62] To date, however, there have been no generally accepted criteria for either hereditary breast cancer, hereditary ovarian cancer, or hereditary breast/ovarian cancer.

- **Criteria for Identifying High-Risk Families**—Several research studies have utilized criteria for identifying high-risk families. These criteria, while using the features of hereditary breast and ovarian cancer as discussed in Table 1, have not been consistent from study to study. In general, these criteria have included cancer in first- or second-degree relatives.

The more widely accepted criteria for identifying high-risk families have been established by Myriad Genetics Laboratory (Salt Lake City, Utah) for use in their beta testing of BRCA1/2 sequencing. In this study, women were eligible if they were diagnosed with either early breast cancer (diagnosed at age < 50 years) or ovarian cancer and had at least one first- or second-degree relative with either early breast or ovarian cancer. Using these uniform criteria, mutations in BRCA1 or BRCA2 were found in 39% of women.[63]

Hereditary breast and ovarian cancers are most likely a heterogeneous set of disorders, and thus, it has been difficult to develop a set of criteria that will identify families that are most likely to carry a BRCA1/2 mutation. The first study to estimate the probability of finding a mutation in BRCA1/2 based on family history was by Couch et al.[64] This study was based on families that presented to high-risk clinics for evaluation. Recent information suggests that the presence of ovarian cancer within a family may be more predictive of a BRCA1 mutation than the number of breast cancer cases in a family.

- **Risk Estimation Models**—Two models for risk estimation—the Myriad and BRCAPRO models[65,66]—have become popular.[63] Using data from 235 women with breast cancer before age 50 or ovarian cancer (at any age) and at least one first- or second-degree relative with either early breast cancer or ovarian cancer, Frank et al were able to develop modeled probabilities of carrying a mutation in BRCA1/2.[63] This Myriad model utilizes extended family history informa-

Table 4

Prevalence of Mutations in BRCA1 and BRCA2 Based on Personal/Family History of Breast Cancer (BC) or Ovarian Cancer (OC)[a]

Personal History	Family History[b]						Prevalence of Mutation BRCA1 or BRCA2	
	No BC at < 50 yr; no OC	BC at < 50 yr in 1 relative; no OC	BC at < 50 yr in > 1 relative; no OC	OC at any age in 1 relative; no BC at < 50 yr	OC in > 1 relative; no BC at < 50 yr	BC at < 50 yr and OC at any age	Persons not of Ashkenazi ancestry	Persons of Ashkenazi ancestry
Breast cancer at ≥ 50 yr	√						1.3%	5.6%
		√					11.9%	8.0%
			√				12.3%	14.3%
				√			4.0%	5.9%[c]
					√		0%[c]	42.9%[c]
						√	25.4%	47.4%[c]
Breast cancer at < 50 yr	√						10.5%	15.2%
		√					19.9%	27.5%
			√				38.3%	43.5%
				√			20.4%	43.6%
					√		30.0%	72.7%[c]
						√	51.1%	58.7%
Ovarian cancer at any age	√						7.0%	15.6%
		√					35.3%	20.0%[c]
			√				39.1%	71.4%[c]
				√			27.5%	40.0%[c]
					√		36.4%	66.7%[c]
						√	41.7%	76.9%[c]

[a] Prevalence of deleterious mutations in either BRCA1 or BRCA2 identified in 5,553 patients undergoing genetic testing through Myriad Genetic Laboratories.

[b] Includes at least one first- or second-degree relative.

[c] n < 20 persons.

Adapted, with permission, from Myriad Genetic Laboratories, Inc, Salt Lake City, Utah (www.myriad.com/gtmp.html).

tion, age at breast cancer diagnosis, and presence of ovarian cancer in the family. As an expansion of this model, mutation prevalence data were made available by Myriad Genetics Laboratories (www.myriad.com/gtmp.html). This information, which is regularly updated, represents individuals who have undergone clinical genetic testing for mutations in BRCA1 and BRCA2 by Myriad Genetics Laboratories. The tables found on this website can be used to estimate the likelihood of finding a mutation in BRCA1/2 based on extended family history, age of breast cancer diagnosis, and ethnicity (ie, Ashkenazi ancestry or not). Table 4 represents a summary of the data on their website.

The second popular model is a computer program called BRCAPRO, developed by investigators at Duke University.[65,66] This model utilizes first- and second-degree family history, presence of breast cancer (male or female) and/or ovarian cancer, age at diagnosis of cancer, ethnicity, and size of the family to estimate the probability of finding a mutation in BRCA1/2. This model is being widely used clinically. A multisite validation study has shown BRCAPRO to be an accurate tool for determining the probability of BRCA1/2 mutations (personal communication, D. Berry, 2000). The BRCAPRO computer program is required when using this model. Further information can be found at www.jhsph.edu/biostats/brcapro.html.

Genetic Testing and Counseling

When deciding to undergo genetic testing, one needs to consider the likelihood of finding a specific germ-line mutation (positive result), as well as the appropriateness of genetic testing for the individual and family; eg, will it change medical management for the individual or other family members? No established guidelines exist for undergoing genetic testing for BRCA1/2. The American Society of Clinical Oncology recommends that a 10% prior probability of BRCA mutation be used as a guideline for offering genetic testing.[67]

As genetic testing for breast and ovarian cancer susceptibility genes has moved from the research laboratory into the clinical setting, interest in this field has increased rapidly. In surveys, 70% to 90% of women with a family history of breast and ovarian cancer expressed interest in undergoing genetic testing. Among first-degree relatives of cancer patients, interest in testing is associated with cancer worry and mood disturbance.[68] There is also a tendency among these individuals to overestimate the benefits and underplay the risks of genetic testing.[69] Women who desire genetic testing are more likely to have a heightened perception of personal risk for being a mutation carrier.

Genetic testing offers a unique method to accurately determine individual risk in families that have an inherited susceptibility to breast and ovarian cancer. Benefits of testing are not universally applicable to all individuals with a family history of cancer. Most women, regardless of their family history, present with the belief that, in terms of their cancer risk, a simple "yes or no" answer will be revealed by testing. The majority of women seek genetic testing as a means to quantify risk for their children and to end uncertainty.[68,70] More importantly, high-risk women might view genetic testing as part of their information-seeking process or as one of their coping strategies.

Thus, the first step in the process must be education. This involves providing information about the basic principles of inheritance and the concepts of inherited and sporadic cancer, along with a personalized assessment of risk and benefits, and limitations of testing as applicable to that individual. These discussions can be time consuming and often require a multidisciplinary approach that includes genetic counselors, medical geneticists, oncologists, and nurses.

Educating the Patient

Studies have shown that genetic counseling has a significant impact on the perception of risk by women who are referred to familial cancer clinics.[53] Combining education with a discussion of psychosocial issues and individual reactions to testing contributes greatly to the changing perception of the risks and benefits of genetic testing.[71]

Genetic testing for mutations that predispose to cancer is not as straightforward as testing for single-mutation diseases, such as sickle cell anemia or Huntington's disease. As a substantial proportion of breast and ovarian cancer is sporadic, more than one family member can be affected by chance. Thus, decisions of whom to test and the interpretation of results are complex and should be part of the discussion before embarking upon genetic testing.

The process of genetic testing should begin with the individual in the family who is most likely to be carrying a germ-line mutation in BRCA1/2. This is always a person with cancer (an affected individual) and is usually the person who has developed cancer at an early age or has an unusual form of cancer (ie, bilateral or multiple primary cancers or rare cancers, such as adrenal cortical carcinoma or osteogenic sarcoma).

Interpretation of genetic test results can be complicated. For the first individuals tested in a family, three types of results are possible:

(1) A deleterious or functional mutation is identified, meaning that the identified mutation is known to interrupt the function of the gene and/or be associated with breast and ovarian cancer in other families. This is a positive result, and this individual has the risk of cancer outlined above and in Table 2. There is now a test to offer others in the family (testing for the specific identified mutation). This test will be either positive (a mutation is identified) or negative (no mutation is identified), and can be performed on both affected and unaffected individuals.

(2) No mutation may be identified. For the first person tested in a family, there are two possible interpretations of this result. If the person is not affected with cancer or has a cancer that is not usually associated with BRCA mutations (such as late-onset breast cancer), then there may still be a mutation in the family, but you have not tested the right person. This is considered a false-negative result. Alternatively, a mutation in BRCA1/2 may not exist in either the individual or the family. Cancer in the latter case may be associated with other genes (not yet identified or not tested for). In either case, recommendations for screening and medical management must be based on the family history.

(3) A mutation of uncertain significance may be identified. In this case, it may not be known whether this mutation is a normal variant of BRCA1/2 (genetic polymorphism) or truly a disease-causing mutation. This test result cannot be used to test others in the family, and recommendations for screening and medical management (discussed below) must be based on the family history.

Limitations of Genetic Testing

Individuals planning to undergo genetic testing must be aware of the limitations of testing. Testing may miss genetic abnormalities in BRCA1/2 that actually exist within a family. For example, this is true if there is a large deletion of the gene. A large deletion will not be picked up through DNA sequencing, because the presence of the normal copy of the gene makes the result appear normal.

As many as 30% of families with breast and ovarian cancer may actually have a germ-line mutation in BRCA1 or BRCA2 that is not identified by conventional genetic testing for these two genes.[13] If no mutation can be identified within a family, it may mean that other genes—not yet identified (for which no genetic test is available)—are operating in that family. A positive test result (a mutation in BRCA1/2) can only provide an estimation of cancer risk, as not all individuals who test positive will actually develop cancer. Recommendations for the management of mutation carriers are largely based on expert opinion (see below). Prior to

undergoing genetic testing, individuals must understand the uncertainty associated with both high-risk cancer screening and options for prevention.

Psychological Distress

The psychological issues involved with testing cannot be underestimated. Discussion of a family history of cancer and the possible implications of genetic tests for other family members, including children, can induce strong emotional responses that need to be dealt with in order to make a balanced decision regarding testing. As discussed earlier, women at high risk often have higher levels of anxiety and psychological distress at baseline. In one study of 60 patients, BRCA1 mutation carriers manifested greater levels of psychological distress 1 to 2 weeks after genetic test results were revealed—even when pretest and posttest counseling was provided.[72] When genetic testing is offered without counseling, it may have an even more negative impact on psychological functioning.

Other studies have shown no significant increase in depressive symptoms in BRCA1 mutation carriers.[73] Feelings of guilt may be an unexpected result of testing among both mutation carriers and noncarriers. Guilt among affected persons often stems from worry of transmitting the gene to children. Among persons who test negative, "survivor guilt" may be observed.[74] Although initially relieved that they do not have high levels of cancer risk, individuals will often feel guilty that they do not have the same risk of cancer as their siblings.

Insurance Discrimination

Fear of insurance discrimination is one of the most cited concerns by patients regarding genetic testing.[70] To address this concern, the Health Insurance Portability and Accountability Act was passed in July 1997. This act prohibits group health insurance plans from using genetic testing results to determine eligibility for coverage or to increase premiums. On a local level, 28 states have enacted laws to prevent the use of presymptomatic genetic testing information by health insurers to discriminate against potential subscribers.

There are no well-documented cases that demonstrate health insurance denial or discrimination on the basis of genetic test results, but these laws have not been adequately tested in our legal system. The choice of the majority of consumers not to disclose genetic testing results to insurance companies or employers without written consent demonstrates the degree of concern with which they view disclosure of this information.[75] Thus, even legal deterrents do not completely allay the fears that individuals have regarding the potential misuse of information obtained from genetic testing.

Effect on Other Family Members

Genetic testing applies to the entire family and not just the tested individual. Testing of one individual has implications for other family members. The mutation status of an untested family member can be revealed through the genetic testing of other relatives. For example, a daughter who tests positive for a mutation found in another maternal relative (aunt or cousin) will reveal the genetic status of her mother, as the mother will be an obligate carrier of that mutation. On occasion, the family member who is most appropriate for testing may refuse to participate, making genetic testing impossible to interpret for other family members. Reviewing the potential impact of genetic testing for the entire family prior to testing may often prevent future problems. Counseling should focus not only on the impact of testing for the immediate family, but on developing a plan for disclosure of results to other family members.

Informed Consent

Counseling is an integral part of the process of obtaining informed consent. The American Society of Clinical Oncology[67]and the National Society of Genetic Counselors[76] have both endorsed the importance of a thorough discussion of the risks, benefits, and limitations of genetic testing prior to signing a written consent form. Certified genetic counselors serving locations across the nation can be identified by accessing the website of the National Society of Genetic Counselors (www.nsgc.org).

Follow-up After Genetic Testing

Following disclosure of test results, counseling involves a discussion of the implications of the results both for the tested individual and other family members. For individuals who test negative when there is a mutation in other family members, it is important to reiterate that the general population risk of breast and ovarian cancer still exists. For persons testing negative without a mutation in the family, it must be stressed that testing does not exclude the possibility of hereditary cancer—especially if there is a strong family history of cancer. For mutation carriers, the focus of discussion centers on high-risk cancer screening recommendations and available management options, which might include chemoprevention or prophylactic surgery (see below). Additional follow-up counseling should be offered either in person or by telephone. A letter detailing the pretest discussion, the test results, and follow-up recommendations is an excellent tool for reference (both for the individual and other family members).

The goal of genetic testing is to provide information that can be used to improve cancer screening and offer management options to women at high risk of breast and ovarian cancer. Several studies have shown that high levels of anxiety can pose a barrier to adhering to cancer screening recommendations.[77] Education and counseling have the potential to empower women at high risk with an understanding of the complex issues that surround genetic testing and to encourage active participation in cancer screening and prevention strategies.

Management of Hereditary Breast and Ovarian Cancer

Women at increased risk of breast or ovarian cancer have several medical options to consider. Expanded cancer screening is frequently offered. Although screening will not prevent cancer, it can diagnose cancer at an earlier stage—one associated with better survival. Prophylactic surgery and chemoprevention using selective estrogen-receptor modulators (SERMs) or oral contraceptives are modalities that have been associated with lowering the risk of breast and/or ovarian cancer. Additionally, issues of hormone replacement therapy must be considered in women at increased risk of breast cancer. We will briefly discuss these

Table 5

Screening Recommendations for Breast and Ovarian Cancer in Women at High Risk

	Age to Start		
	BRCA1/2 Mutation Carrier[a]	High-Risk Individual/Family	Screening Interval
Breast Cancer Screening			
Breast self-examination	20–25 yr	20–25 yr	Monthly
Clinical breast examination	25–35 yr	25–35 yr	Twice-yearly
Mammogram	25 yr	5–10 yr prior to youngest breast cancer diagnosis in family	Yearly
Ovarian Cancer Screening			
Pelvic examination	> 18 yr	> 18 yr	Yearly
Transvaginal ultrasound	25–35 yr	25–35 yr	Every 6–12 mo
CA-125	25–35 yr	25–35 yr	Every 6–12 mo

[a] Adapted from Burke et al.[81]

issues, as well as discuss the implications of a strong family history of breast and ovarian cancer in women with newly diagnosed breast cancer.

Breast Cancer Screening in Women at High Risk

Women who have a greater-than-average risk of either breast or ovarian cancer are candidates for increased cancer surveillance. Cancer screening recommendations are often tailored to the family history and to the individual's personal risk of breast or ovarian cancer. The benefits of screening for women at high risk have not been proven in clinical trials. When making recommendations for increased cancer surveillance in either BRCA1/2 carriers or women at increased risk, it is important to discuss the limitations of screening, as well as the unproved nature of screening recommendations.

Screening mammography has been associated with improved survival for women over age 50.[78,79] Women between the ages of 40 and 49 also benefit from mammography, but that benefit is smaller.[80] More frequent breast cancer screening is recommended for women who are found to carry BRCA1/2 mutations.[81] Recommended screening includes monthly breast self-examination, twice-yearly clinical breast examination, and yearly mammography starting at age 25 (see Table 5). These recommendations are based on expert opinion.[81]

Emerging data suggest that early mammography for women at high risk may be beneficial and associated with an earlier stage at diagnosis.[82] Most women with a family history of breast and/or ovarian cancer will not undergo genetic testing, or if they do undergo testing, no mutations will be found in the family. Increased cancer surveillance seems prudent for these women, although screening recommendations often differ from center to center.[83]

It is generally recommended that women at higher-than-average risk of breast cancer perform monthly breast self-examination and undergo twice-yearly clinical breast examination. Yearly mammography is also recommended for women at greater-than-average risk. Mammography should be initiated 5 to 10 years prior to the youngest diagnosis of breast cancer in the family, but not before age 25. Women in high-risk families should be encouraged to have any lump evaluated promptly and thoroughly. Recent information supports the use of breast ultrasound for the evaluation of high-risk women.[84] See Table 5 for recommendations for high-risk women.

Ovarian Cancer Screening in Women at High Risk

High-risk screening for ovarian cancer is a controversial subject. No techniques have shown high sensitivity and specificity for detection of ovarian cancer.[85,86] Some studies suggest that screening women at increased risk is associated with higher cancer identification rates than in the general population.[87] Ovarian cancer screening is recommended for women who are found to carry mutations in BRCA1/2. These recommendations are supported by expert opinion.[81]

Additionally, increased surveillance is often recommended for women who have a first- or second-degree relative with ovarian cancer. Recommendations include annual pelvic examination, annual or semiannual CA-125 measurement, and transvaginal ultrasound. These recommendations rely on the fact that some studies have demonstrated improved screening efficacy when both techniques are used.[88,89] Women should understand that CA-125 levels are higher for premenopausal women (compared with postmenopausal women) and can be elevated due to other benign and malignant conditions such as pregnancy, endometriosis, pelvic inflammatory disease, hepatitis, and colon cancer. Furthermore, false-positive tests may result in exploratory surgery.

Chemoprevention in Women at High Risk

- **Chemoprevention for Breast Cancer**—Women at increased risk of breast cancer may be candidates for chemoprevention. Three trials of the SERM tamoxifen (Nolvadex) for the prevention of breast cancer have been conducted. The largest and most recently completed trial showed a 50% reduction in the development of inva-

sive breast cancer among women taking tamoxifen for 5 years.[90] This trial, performed by the National Surgical Adjuvant Breast and Bowel Project (NSABP), randomly assigned women at increased risk of breast cancer (based on age, hormonal factors, family history of cancer, or personal history of a breast biopsy) to either tamoxifen or placebo. Significant reductions in both invasive and noninvasive breast cancers were seen for all groups of women in the study. However, the reduction in invasive breast cancer was limited to estrogen receptor–positive tumors.

These results need to be interpreted with caution in high-risk individuals. Women with hereditary breast cancer tend to develop hormone receptor–negative breast cancer.[91,92] Therefore, there is concern that women with a strong family history of breast cancer may not achieve the same benefit from tamoxifen.

Two other clinical trials of tamoxifen in women at increased risk of breast cancer have been performed. Neither of these studies showed any benefit of tamoxifen for women at increased risk.[93,94] Although both of these studies were smaller and had different eligibility criteria, they were well designed and had longer follow-up periods than the NSABP P1 trial.[90,93,94] The Royal Marsden trial was performed in women with a family history of breast cancer.[93]

The SERM raloxifene (Evista) may turn out to be another alternative for women at risk of breast cancer. Two studies have shown reductions in the incidence of breast cancer in women taking this drug.[95,96] Again, caution is warranted, as these two trials used only postmenopausal women and were primarily performed to investigate the role of raloxifene in the treatment of osteoporosis.

• **Chemoprevention for Ovarian Cancer**—Chemoprevention for ovarian cancer is available. Studies have shown that women who are at average or increased risk of ovarian cancer benefit from treatment with oral contraceptives.[97-99] A 60% reduction in the risk of ovarian cancer was seen for women with BRCA1/2 mutations who used oral contraceptives for more than 6 years.[99]

Long-term, uninterrupted use of oral contraceptives is associated with a slight increase in the incidence of breast cancer.[100] This risk may be greatest for women with a family history of breast cancer.[101] For women at increased risk of ovarian cancer or those who have a great likelihood of carrying a germ-line mutation in either BRCA1 or BRCA2, the benefits (reduction in ovarian cancer risk) need to be weighed against the slightly increased risk of breast cancer.

Surgical Prophylaxis in Women at High Risk

Women at the greatest risk of breast and/or ovarian cancer may consider the prophylactic removal of breast or ovarian tissue. Women who undergo prophylactic mastectomy can reduce their risk of breast cancer by 90%.[102] Satisfaction is generally high, and quality of life may be improved by prophylactic mastectomy for high-risk women.[103] Prophylactic oophorectomy has also recently been shown to reduce the risk of ovarian cancer by 90%.[104] Additionally, prophylactic oophorectomy in women who carry BRCA1/2 mutations has been shown to decrease the risk of breast cancer by at least 50%.[105]

For high-risk women and their caregivers, these are complex issues. While a prophylactic oophorectomy may reduce the risk of both breast and ovarian cancer, the consequences of a lifetime lack of estrogen must be considered. For women who undergo early prophylactic oophorectomy, some would advocate the use of hormone replacement therapy until age 50.[106] These women should be closely monitored for changes in bone density and cholesterol.

Hormone Replacement Therapy in Women at High Risk

The use of hormone replacement therapy for women in high-risk families is also a complex issue. Women and their primary caregivers are often reluctant to initiate hormone replacement therapy in women with an elevated risk of breast cancer. It is known that women on prolonged hormone replacement therapy (> 4 years) have a slightly increased risk of breast cancer.[107] Risk also appears greater for women with a family history of breast cancer. However, survival is no different in the two groups.[108]

Two recent studies suggest that, for all women. the combination of estrogen and progestin may be associated with a greater risk of breast cancer. compared to estrogen alone.[109,110] For any woman, the risks and benefits of hormone replacement therapy need to be considered; it may be prudent to limit the amount of progesterone exposure, especially for women at increased risk of breast cancer. All women on hormone replacement therapy should have yearly mammograms and be encouraged to perform breast self-examination in conjunction with yearly or twice-yearly clinical breast examination.

Second Primary Breast Cancer in Women at High Risk

The surgical treatment of breast cancer has become less aggressive over time. Women often have the option of undergoing lumpectomy, axillary evaluation, and radiation therapy (breast-conserving therapy) or mastectomy as primary treatment for their breast cancer. For women with either a family history of breast cancer[111-113] or a BRCA mutation,[114] there is no increase in local recurrence with breast-conserving therapy. There is, however, an increased risk of developing a second primary breast cancer for both groups.[113-116]

Second primary breast cancers may be seen in a different quadrant of the same breast, or more commonly, in the contralateral breast. The studies cited above are not large, but they suggest that women with a family history of breast cancer or BRCA mutation can be safely treated for their primary breast cancer with breast-conserving therapy. Nevertheless, a woman should consider her risk of a second breast cancer and prophylactic surgery at the time of a first breast cancer diagnosis. Of course, this can be a difficult decision to make at the time of diagnosis, and there is no harm in delaying this decision until the completion of the primary breast cancer therapy.

Conclusions

Women with a strong family history of breast and ovarian cancer have several options to consider. Only a minority of individuals with a strong family history will be candidates for

genetic testing for BRCA1/2, and mutations will be found in even fewer persons. Candidates for genetic testing need to understand the risks, benefits, and limitations of genetic testing prior to testing.

The risk of cancer for individuals with a strong family history of breast and/or ovarian cancer makes high-risk screening a prudent consideration. Benefits of screening for this population are not clear. Options for chemoprevention in high-risk individuals are also available. Prophylactic surgery (mastectomy or oophorectomy)—although an irreversible and life-altering decision—offers the greatest reduction in cancer risk.

We have much to learn in this rapidly developing field. Women who are at high risk may be candidates for clinical trials and/or research studies, and should be informed of these options when they are available.

This article is reviewed on pages 902, 906, and 912.

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The Srivastava/McKinnon/Wood Article Reviewed

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The article by Dr. Srivastava and colleagues provides a broad overview of the procedures for assessing the risk of breast and ovarian cancer in women with a family history of these diseases. Below, we make some additional comments that may be useful to practitioners in risk-evaluation clinics.

Founder Mutations

When evaluating patients for possible deleterious BRCA mutations, determining ancestry may be a critical issue. Founder mutations are present in ethnically isolated populations and are responsible for a significant proportion of breast and ovarian cancer cases attributed to an inherited susceptibility. Founder mutations have been identified in Ashkenazi Jews,[1-3] French Canadians,[4] Japanese,[5] Italians,[6] Swedes,[7] Finns,[8] Belgians, and Dutch.[9,10]

In Caucasian individuals, it is estimated that 1 out of 300 individuals carries a mutation in BRCA1 or BRCA2, whereas 1 out of 40 individuals of Ashkenazi Jewish ethnicity harbors such a mutation. Penetrance of BRCA1/2 may be somewhat lower in Ashkenazi Jews than estimates derived from studies of the original families[11]; however, other recent studies show a higher penetrance. Individuals of Dutch ancestry may be advised to pursue a special test that is designed to detect two founder genomic deletions that have been observed in this population.

Genes That Predispose to Breast Cancer

Variants of uncertain significance (VUS) abound in the BRCA genes. Their significance remains uncertain primarily due to the relatively scant knowledge regarding their biological impact. Testing additional family members for the familial VUS can be helpful in establishing, for example, that the VUS was inherited from a noncancer lineage or that the VUS does not appear to track with cancer cases seen in the family.

The BRCA genes are examples of tumor-suppressor genes as defined by Knudson's two-hit hypothesis. As the authors point out, other tumor-suppressor genes such as p53 (one of the genes responsible for Li-Fraumeni syndrome) also predispose to hereditary breast cancer. Li-Fraumeni syndrome (or SBLA syndrome [sarcomas, breast and brain tumors, leukemia, laryngeal and lung cancer, and adrenocortical carcinoma]) can also be caused by mutations in the newly discovered hCHK2 gene, which confers a predisposition to sarcoma, breast cancer, and brain tumors.[12] It is anticipated that testing for this gene in certain families may be appropriate when such a test becomes clinically available.

The Best Model

When assessing empiric risk for breast cancer, it is best to use the model that shows the fewest limitations for the patient's personal and family history. In contrast to the authors' statement, the Claus model does provide

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estimates of breast cancer risk for women with a family history of ovarian cancer in a first-degree relative.[13] The BRCAPRO model is limited in that it considers BRCA1/2 to be the only possible predisposing genes, with all other high-penetrance breast cancers being scored as sporadic. Some scientific evidence points to other breast cancer susceptibility genes that have yet to be identified, and therefore, BRCAPRO is likely to overestimate the probability of a BRCA mutation in a family. This model also fails to incorporate a previous breast tissue diagnosis that may impact risk.[14] When calculating the risk of finding a BRCA mutation in a family, the uncertainty is even greater.

The Myriad tables should be used with caution, because the informed consents used by many clinics may not allow for the dissemination of family history information to the testing company. All models are meant only as guides for the counselor and patient, setting the stage for a risk-evaluation discussion tailored to the patient's needs.

Although patients considering risk management options are often referred to practitioners in other disciplines, it is neither time efficient nor cost effective to have onsite multidisciplinary specialists. The model of a risk evaluation clinic run by a genetic counselor or nurse geneticist plus a physician versed in cancer genetics is widely adopted and appears to work quite well.

Treating Women at High Risk for Breast Cancer

The earliest steps in breast carcinogenesis due to BRCA1/2 mutations are not completely understood. More information is needed prior to making any definitive statements on genotype/phenotype correlations. A preponderance of estrogen receptor–negative tumors may apply to BRCA1-related disease, but not cancer associated with BRCA2. Regarding the use of selective estrogen-receptor modulators for chemoprevention, we believe that raloxifene (Evista) is not yet a risk-reduction alternative for high-risk breast cancer patients outside of research protocols.

Breast conservation appears to be a reasonable option for most women who are BRCA carriers. Nevertheless, women who are mutation carriers and require mastectomy to treat a breast cancer diagnosis may wish to discuss the risks and benefits of a possible bilateral prophylactic mastectomy, if they desire reconstruction with autologous tissue after their therapeutic mastectomy, as this type of reconstruction can, in general, only be performed once with present techniques.

With or without reconstruction, prophylactic bilateral mastectomy is an option that many BRCA1/2 carriers wish to discuss. The usefulness of this risk management option is also controversial. A retrospective study by Hartmann et al revealed a 90% reduction in breast cancer risk after prophylactic mastectomy.[15] However, it is not known how many of the women studied actually had a BRCA1/2 mutation. Inclusion criteria for "high-risk" women were broad and included variables that may not be associated with an inherited susceptibility to breast cancer. Therefore, the women in this cohort who underwent prophylactic mastectomy may not have been at "high" enough risk. Furthermore, there are almost no data on any long-term effects, such as quality of life and psychosexual function. These results must be applied with caution to the BRCA carrier population until further genetic information becomes available.

Controlling Breast Cancer Risk

We believe that significant scientific evidence supports certain lifestyle interventions to control breast cancer risk. For example, limiting alcohol to three to five drinks per week,[16] consumption of more than five servings of fruits and vegetables per day,[17] and routine exercise[18,19] have all shown substantial benefits with regard to breast cancer risk. It is reasonable to consider counseling on these measures as part of a patient's breast cancer risk management.

The Health Insurance Portability and Accountability Act is a major step forward in protecting patients from genetic discrimination. However, detailed knowledge of its specific limitations is helpful for certain patients. The protection provided by this law does not extend to life and disability insurance, and we believe these issues should be discussed with patients, especially young patients who may not have life and disability insurance.[20]

Conclusions

In practical terms, there is a pressing need to more fully understand the efficacy of lifestyle changes, as well as chemopreventive and surgical interventions, as they apply to carriers of specific gene mutations. In particular, appropriate end points and patient satisfaction should be studied, in addition to cancer risk and survival. For example, research outcomes that center on patient desires, satisfaction, and decision-making processes are largely unknown for these high-risk individuals. Integrating this knowledge into a more detailed understanding of hormone action on breast tissue will be needed to design effective and comprehensive cancer risk reduction interventions for women at high risk.

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The Srivastava/McKinnon/Wood Article Reviewed

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Assessing genetic risk for women with strong family histories of breast and ovarian cancers is becoming increasingly important in oncology and medical genetics as our understanding of the molecular basis of these cancers improves. Dr. Srivastava and coauthors outline some of the major features of genetic counseling for these malignancies. Many of these principles apply to genetic counseling in general and are not unique to women with an increased risk of breast and ovarian cancer. We encourage all physicians to inquire about family history on both the maternal and paternal sides and to refer any patient (woman or man) whom they suspect may be part of a familial syndrome for more specialized counseling.

Family History Issues

Defining what constitutes a strong family history is difficult, because many people today are members of small nuclear families or may not be in contact with relatives. Paternal transmission is often ignored in breast cancer risk assessments, but can be relevant in cases of paternal transmission where there may be no first-degree relative with breast or ovarian cancer. Therefore, the strongest indicator of a heritable syndrome of breast and ovarian cancers may be the presence of early-onset disease or multiple primary tumors within an individual.

Although the likelihood of finding a deleterious mutation in a susceptibility gene increases with the number of affected individuals in a family, mutations have been reported in patients who do not report any family history of breast or ovarian cancers. This may be due to numerous factors, including patients being unaware of family members' medical conditions, as well as the presence of low-penetrance alleles of cancer susceptibility genes.

Translating Complex Data for Patients

Clinical encounters with patients seeking genetic testing are challenging in that a tremendous amount of sophisticated information must be conveyed in simple language to patients who are often anxious and may have limited previous education in genetics. Discussions with patients are based on the most complete and accurate family history that is attainable and include education in the major heritable predisposition syndromes, lifetime risk assessment, likelihood of finding a deleterious mutation, and an outline of risk-reducing options.

Obtaining the most accurate and complete family history is the key to risk assessment. All disease states or associated conditions should be noted and pathology reports should be used to substantiate cancer diagnoses. This latter recommendation is tedious, but exceedingly important since the family tree is used to decide the likelihood of a genetic predisposition as well as which individuals would be expected to be the most informative for genetic testing. Diagnoses in generations older than the patient seeking risk assessment tend to be the most vague, and confirmation is critical.

Multiple visits with numerous family members may be necessary to adequately explain the issues of chromosomal inheritance, predisposition genes, and subtleties within the field, such as the distinction between polymorphisms and mutations. Patients are often asked to reiterate what has been outlined for them, in an effort to ensure understanding. Patients may need to discuss these issues with their cancer care specialist, who should be knowledgeable about the complexities of genetic testing.

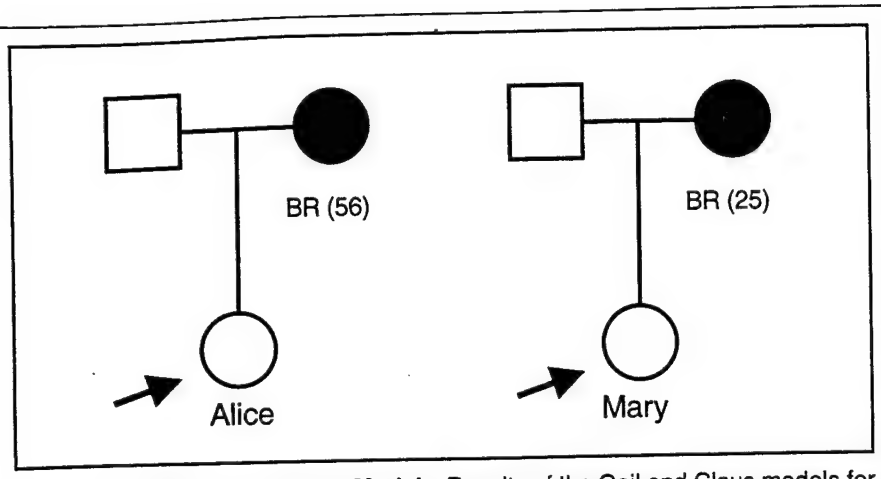

Figure 1: Gail Model vs Claus Model—Results of the Gail and Claus models for cancer risk can differ significantly, depending on family history. In this case example, Mary and Alice came in for risk-assessment counseling. Both were 35 years old, both had menarche at age 14, had no children, and had never had a breast biopsy. Both had mothers in whom breast cancer had been diagnosed: Mary's at age 25, Alice's at age 56. Each woman wanted to know her risk of developing breast cancer.

By the Gail model, both have the same lifetime risk of 15.3%. By the Claus model, Alice's lifetime risk is 11.0% and Mary's is 21.1%. This demonstrates the fact that the Gail model does not take into consideration age at diagnosis. Mary learns that her maternal aunt was diagnosed with breast cancer at age 43. Mary's lifetime risk estimate using the Claus model then increases to 40.7%, although this is not reflected by the Gail model.

Flaws in Risk Hierarchy

Numerous hereditary syndromes, inherited in an autosomal dominant fashion predispose to the development of breast and ovarian cancers, and the responsible genes are known in most cases: breast/ovarian cancer syndrome (BRCA1/BRCA2); Cowden's syndrome (PTEN); Peutz-Jeghers syndrome (LKB1/STK11); Muir-Torre syndrome (MSH2/MLH1); and Li-Fraumeni syndrome (TP53). Srivastava et al divide these syndromes into three categories (high, moderate, and low) based on the likelihood that the patient with that syndrome will develop breast or ovarian cancer. This simple categorization is misleading, however.

Mutations in genes such as PTEN confer a high risk but are variably penetrant, probably based on other modifying genes.[1] Therefore, to place these syndromes in such a hierarchy implies that patients with Cowden's syndrome, for example, are at a lower risk for the development of breast cancer, compared to a woman with a BRCA2 mutation. However, depending on the specific mutation present within a family and the accompanying modifying genes within an individual, this may not be true. Therefore, hereditary syndromes that confer an increased risk of breast and ovarian cancer should be considered equally within families.

On the other hand, there are probably other genes with low-penetrant alleles that contribute to increased breast cancer risk in families. However, such genes should probably not be classified as "low-risk" genes. Assessment of risk should be made on an individual basis. One of the limitations of genetic testing at the present time is that we are only offering testing for coding region mutations in BRCA1 and BRCA2.

Limitations of Genetic Testing

Individual risk assessment for breast and ovarian cancers is a complex issue. Risk can be estimated in two ways. First, computer models exist to approximate the likelihood of a patient developing breast or ovarian cancer within her lifetime and to calculate the likelihood of a patient having a BRCA1 or BRCA2 mutation. Second, genetic testing can be performed to determine whether an individual carries a deleterious mutation in one of the genes known to predispose to breast and ovarian cancer.

The lifetime risk of a patient developing breast or ovarian cancer can be approximated using the Gail or Claus models, each of which is useful but has limitations (Figure 1).[2,3] The Gail model considers the following risk factors: age of onset of menarche, age at first birth, the number of first-degree relatives with breast cancer, and the number and outcomes of breast biopsies. The Gail model does not take into account ages at cancer diagnosis or relatives beyond the first degree (thereby almost assuredly neglecting paternal transmission of a mutation).

In contrast, the Claus model considers breast cancer cases within first- and second-degree relatives as well as ages at diagnosis, but may overestimate risk in nonmutation carriers. Figure 1 demonstrates an example in which the Gail and Claus models yield significantly different risk estimates. In clinical practice, both estimates can be helpful in counseling and should be calculated.

The likelihood that a patient carries a mutation in BRCA1 or BRCA2 can be estimated using the BRCAPRO[4,5] or Myriad models.[6] Variables considered in the BRCAPRO calculation include first- and second-degree relatives with breast/ovarian cancers, cancers in males vs females, ages at diagnosis, ethnicity, and family size. The Myriad model considers the extended family history, ages at diagnosis, and ethnicity. In general, these two models generate similar estimates and may be helpful in identifying patients who are likely to benefit from BRCA1/BRCA2 genetic testing, which now costs over $2,600.

Taken together, these two types of risk assessments are very important when counseling patients. Patients with strong family histories generally overestimate their risks of developing breast or ovarian cancers.[7] Providing these risk approximations for patients can significantly ease anxiety, may affect a patient's decision to un-

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dergo testing for BRCA1/BRCA2 mutations, and/or may alter a patient's perspectives on risk-reduction strategies. The most common reason patients are referred to our cancer risk clinic at the University of Chicago is to discuss options for risk reduction, especially as they approach menopause and have to decide on hormone replacement therapy.

Reducing the Risk for Breast and Ovarian Cancers

Options to decrease a patient's risk of developing breast or ovarian cancer fall into three general categories: increased surveillance, chemoprevention, and prophylactic surgery. The first entails encouraging patients to perform a monthly breast self-examination and to undergo twice-yearly medical examinations and yearly mammography. In patients with strong family histories, screening mammography should begin 5 to 10 years prior to the age at which the youngest woman in the family was diagnosed with cancer, but probably not before age 25. Newer techniques for breast imaging, such as digital mammography, breast ultrasound, and magnetic resonance imaging, are modalities that are likely to become more frequently used in the coming years.[8]

Screening for ovarian cancer is more controversial, and therefore, when appropriate, patients should be encouraged to participate in clinical trials. For women known to carry a mutation in BRCA1 or BRCA2, a semiannual pelvic examination with transvaginal ultrasound is recommended. Some advise following a patient's CA-125 level semiannually as well.

Women with strong family histories of breast cancer are candidates for chemoprevention. The use of oral contraception agents has been shown to be an effective prevention strategy in protecting women against ovarian cancer.[9-11] Tamoxifen (Nolvadex) reduces the risk of developing breast cancer in high-risk women by close to 50%, but the effect in BRCA carriers is unknown.[12] In addition, women who carry BRCA1 or BRCA2 mutations and have had breast cancer can decrease their risk of developing cancer in the contralateral breast by about 50% with 5 years of tamoxifen therapy.[13] Whether other selective estrogen-receptor modifying drugs will have similar effects is currently being tested. The Study of Tamoxifen and Raloxifene (STAR) is randomizing 22,000 high-risk postmenopausal women to either tamoxifen or raloxifene (Evista) and will follow them to determine which drug is more effective at preventing breast cancer.

Because patients with strong family histories tend to overestimate their risk of developing breast or ovarian cancers, they also tend to refuse estrogen-replacement therapy after menopause. However, the morbidity associated with estrogen depletion in postmenopausal women far outweighs the small increased cancer risk associated with hormone replacement therapy. Therefore, patients who are not considered at high risk after careful risk assessment should be encouraged to adopt effective strategies to reduce their risk of osteoporosis and cardiovascular disease because cardiovascular disease remains the leading cause of death for women after menopause.

Prophylactic surgery is the most radical option for women at increased risk due to strong family histories of breast and ovarian cancers. Most women who carry BRCA1 or BRCA2 mutations do not choose to undergo bilateral mastectomies, but those who do remain content with their decisions several years later.[14] For women who have completed their families, prophylactic oophrectomy almost entirely relieves them of the risk of ovarian cancer and significantly decreases their risk of breast cancer.[15]

Women who seek surgical consultations without having had comprehensive genetic counseling may benefit from hearing of their risks and options prior to undergoing surgery for many of the reasons outlined above. Before a woman undergoes prophylactic surgeries (without knowing the mutation status of her BRCA1 and BRCA2 genes), it is essential that she have a firm understanding of her quantitated risks.

Conclusions

Medicine today is becoming increasingly molecular in its diagnosis and treatment. Because of the numerous hereditary syndromes and known genes that predispose to the development of breast and ovarian cancers, women with strong family histories need to be identified and counseled by a multidisciplinary team. Their risks and options need to be outlined. Such women and families need to be encouraged to participate in clinical trials to evaluate the psychological implications of these diseases as well as our therapeutic interventions.

Genetic testing for cancer susceptibility is here to stay. Oncologists should now embrace the translation of these scientific advances to clinical care.

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—Olufunmilayo I. Olopade, MD

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The Srivastava/McKinnon/Wood Article Reviewed

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The review of hereditary breast and ovarian cancer by Dr. Srivastava et al in this issue of *Oncology* represents an excellent attempt to summarize the state of an increasingly complex field. The authors have an active program in cancer risk assessment and genetics at the University of Vermont in Burlington, and their experience is apparent as they organize the burgeoning data on risk assessment and management of carriers of BRCA1 and BRCA2 mutations.

Testing an Affected Family Member

The full set of issues facing women with hereditary breast or ovarian cancers could not be addressed in detail in such an overview. A cancer diagnosis is often the event that prompts the recognition that a hereditary predisposition might be present in a family. Until all genes and their risk-conferring mutations are known, the preferred strategy for evaluating a kindred for a possible hereditary predisposition remains the initial genetic testing of an affected family member. Ideally, this would be the affected family member who is most likely to carry a mutation based on age at diagnosis (younger rather than older), precise diagnosis (medullary breast cancer or papillary serous ovarian cancer), and position in the pedigree. These characteristics are considered because of the possibility of phenocopies for a common disease like breast cancer.

Even in a family with a documented mutation, sporadic breast and ovarian cancers can occur. Available models consider this troubling issue by assigning higher prior probabilities of carrying a mutation to women diagnosed at younger ages, men with breast cancer, and women diagnosed with both breast and ovarian primary malignancies. A negative genetic analysis in a woman diagnosed with breast cancer at age 55 should not necessarily be the end of the evaluation of a kindred in whom early-onset breast and ovarian cancers cluster elsewhere.

Effect of BRCA1/2 Findings on Management

As noted by Srivastava et al, knowledge of a deleterious BRCA1 or BRCA2 mutation is beginning to influence the management of newly diagnosed breast cancers in mutation carriers. Demonstration of the increased risk of second primary cancers in mutation carriers, and the effectiveness of prophylactic mastectomy, have led women and their physicians to more carefully consider the risks and benefits of breast-conserving therapy compared to bilateral mastectomy for primary treatment. Factors that must also influence these decisions include the age of the woman at diagnosis (since younger women stand to gain more life expectancy) and overall prognosis (as women must survive their first tumor in order to benefit from preventing a second cancer.)[1] The model by Schrag et al also emphasizes the importance of an accurate genetic analysis for women with cancer, in its estimation of additional survival benefits from prophylactic contralateral mastectomy of only 2 to 4 weeks for women without predisposing mutations.[1]

Similar clinical considerations influence decisions for women who recognize their hereditary risk after completion of initial cancer treatment. For many breast cancer survivors, the identification of an increased risk of ovarian cancer associated with a BRCA1 or BRCA2 mutation is an important consideration in the decision to undergo genetic testing. Given mounting concerns about the insensitivity of available ovarian screening methods, and the growing evidence of the effectiveness and comparatively low complexity of laparoscopic ovarian surgery, prophylactic oophorectomy is frequently the first action taken by women learning of their mutation carrier status. Women who have had breast cancer are generally precluded from using hormone replacement therapy, a factor that may influence their decisions about the timing of oophorectomy.

Ovarian cancers that occur among mutation carriers have been shown to carry better prognoses than similar sporadic tumors.[2] Whether BRCA1/2-associated breast cancers respond differently to standard treatments than sporadic tumors remains a controversial issue.[3] The recent demonstration that the microarray appearances of BRCA1/2-associated breast cancers differ not only from sporadic cancers, but also from each other, may have important implications for future tai-

lored therapies, as well as upcoming analyses of risk and outcome data for the two genes.[4]

Breast and ovarian cancer survivors often underestimate the potential impact of learning that they carry a predisposing mutation that may account for their malignancy.[5] Women who have had cancer are therefore likely to benefit from genetic counseling as they consider not only whether to have testing, but also when such information would be most useful to them and their families. Many insurers have begun to pay for genetic testing for women who meet specific criteria, and to cover prophylactic surgical procedures as well. However, fewer insurers are willing to pay for testing in women with metastatic disease, whose information may have a more direct application in the management of risk in their relatives, rather than their own cancer care.

Conclusions

Srivastava et al have provided a comprehensive review of issues in the recognition and management of hereditary breast and ovarian cancer risk. Similar efforts to provide data for other strong cancer predisposition genes will be necessary as new genes continue to be identified for these and other malignancies. The paradigms highlighted in this article are likely to be applied repeatedly as the clinical implications of these genes are elucidated.

—Judy Garber, MD, MPH

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Mitogen activated protein kinase pathway is involved in RhoC GTPase induced motility, invasion and angiogenesis in inflammatory breast cancer

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Abstract

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer known. IBC carries a guarded prognosis primarily due to rapid onset of disease, typically within six months, and the propensity of tumor emboli to invade the dermal lymphatics and spread systemically. Although the clinical manifestations of IBC have been well documented, until recently little was known about the genetic mechanisms underlying the disease. In a comprehensive study aimed at identifying the molecular mechanisms responsible for the unique IBC phenotype, our laboratory identified overexpression of RhoC GTPase in over 90% of IBC tumors in contrast to 36% of stage-matched non-IBC tumors. We also demonstrated that overexpression of RhoC GTPase in human mammary epithelial (HME) cells nearly recapitulated the IBC phenotype with regards to invasion, motility and angiogenesis. In the current study we sought to delineate which signaling pathways were responsible for each aspect of the IBC phenotype. Using well-established inhibitors to the mitogen activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways. We found that activation of the MAPK pathway was responsible for motility, invasion and production of angiogenic factors. In contrast, growth under anchorage independent conditions was dependent on the PI3K pathway.

Abbreviations: ELISA – enzyme linked immunoabsorbant assay; FBS – fetal bovine serum; FGF2 – basic fibroblast growth factor; FGF-BP – fibroblast growth factor binding protein; HME – human mammary epithelial; IBC – inflammatory breast cancer; IGFBP-rP – insulin-like growth factor binding protein related protein; IL – interleukin, LABC – locally advanced breast cancer; MAPK – mitogen activated protein kinase; MEM – minimal essential medium; MTT – 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PI3K – phosphatidylinositol-3 kinase; TCA – tricholoracetic acid; VEGF – vascular endothelial growth factor

Introduction

Inflammatory breast cancer (IBC) is a phenotypically distinct form of locally advanced breast cancer (LABC) that has a propensity to invade, grow and spread in the dermal lymphatics of the skin overlying the breast [1–3]. It is the ability of the tumor emboli to invade and block the dermal lymphatics that leads to its poor prognosis [1–4].

Although the clinical manifestations of IBC have been well documented in the literature, until recently little was known about the molecular mechanisms involved in conferring the unique IBC phenotype. In an effort to identify genetic alterations involved in determining the IBC phenotype, our laboratory isolated two genes that were consistently and concordantly altered in IBC compared with stage-matched non-IBC tumors [5]. RhoC GTPase, was found to be over-expressed in 90% of IBC tumors versus 36% of the stage-matched controls.

RhoC GTPase is a member of the Ras-superfamily of small GTP binding proteins and is primarily responsible for re-organization of the actin cytoskeleton leading to the formation of lamellipodia and fillipodia resulting in cellular motility [6–13]. Transfection of the RhoC homologue, RhoB, into Ras-transformed NIH3T3 cells leads to increased focus formation suggesting a role for the Rho proteins as a transforming oncogene or as a metastasis gene [14]. Similarly, our laboratory has demonstrated that RhoC transfected HME cells become highly motile and invasive, grow under anchorage independent conditions, produce angiogenic factors, and are tumorigenic and metastatic when orthotopically implanted into nude mice [15–17].

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These *in vitro* data have additional clinical significance as RhoC overexpression is associated with the transition to metastatic disease in other cancers [18–20]. It has been proposed that Rho proteins act through and potentiate signaling via the c-Jun kinase/stress activated protein kinase (JNK/SAPK) and mitogen activated protein kinase (MAPK) pathway (reviewed by Takai et al. [21]). Evidence from other laboratories suggest that Rho proteins can signal through both the MAPK pathway as well as the phosphoinositol-3 kinase (PI3K) pathway, while cdc42 and Rac1 are associated with the JNK/SAPK pathway [22–25]. Furthermore, it has been demonstrated that in certain cell types, the MAPK pathway is involved in signaling and the production of angiogenic factors while the PI3K pathway is involved in growth and survival [26–33].

In the present study we set out to determine the major pathways involved in RhoC signaling in IBC. Specifically, we attempted to determine which pathways and cascades were involved in conferring specific aspects of the RhoC-induced phenotype. Many of the published studies that describe the signal transduction pathways involved in Rho signaling were performed in transfected NIH3T3 cells, thus our study focused on the RhoC signaling pathways specific to IBC and HME cells. We treated HME-RhoC stable transfectants, control HME-β-galactosidase (HME-β-gal) transfectants or the SUM149 IBC cell line with C3 exotransferase (a specific inhibitor of Rho proteins), a variety of MAPK inhibitors, or a PI3K inhibitor and assayed them for specific biological functions. The inhibitors were used at concentrations that would inhibit signal transduction without affecting cellular viablility. We found that the PI3K pathway was involved in anchorage independent growth and survival, while multiple arms of the MAPK pathway were involved in motility and invasion, and that p38 is a downstream modulator in the production of angiogenic factors. These data provide significant new insight as to how overexpression of RhoC can lead to a variety of phenotypic effects in breast cells.

Materials and methods

Cell culture

Cell lines were maintained under defined culture conditions for optimal growth in each case [34–36]. Briefly, human mammary epithelial (HME) cells were immortalized with human papilloma virus E6/E7 [37] and grown in 5% fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, Missouri) supplemented Ham's F-12 medium (JRH BioSciences, Lenexa, Kansas) containing insulin, hydrocortisone, epidermal growth factor, and cholera toxin (Sigma Chemical Co.). Stable HME transfectants containing either the human wild-type RhoC GTPase or control β-galactosidase genes were produced and maintained in the described medium supplemented with 100 μg/ml hygromycin (LifeScience Technologies) as previously published [15–17]. The SUM149 cell line was developed from a primary IBC tumor and grown in 5% FBS supplemented Ham's F-12 medium containing insulin and hydrocortizone. The HME cells were characterized as being keratin 19 positive, ensuring that they are from the same differentiation lineage as the SUM149 IBC tumor cell line [5].

Cells actively growing in culture were treated with MAPK inhibitors. 2.0 μM PD98059, 1.5 μM U0126, 1.5 μM SKF86002, or 1.5 μM SB220025 (all obtained from Calbiochem, San Diego, California) 24 h prior to assays and treated everyday with fresh inhibitor until the end of the assay. Treatment of cells with 2.5 μM LY294002 (Calbiochem), a PI3K inhibitor, was performed in the same manner as described for the MAPK inhibitors. These concentrations were below the IC_{50} of the compounds to avoid direct cell toxicity to allow for meaningful biological assays.

Western blot analysis

Proteins were harvested from cell cultures using RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 1 mM sodium orthovanadate and 0.3 mg/ml aprotinin; Sigma Chemical Co.). Ten μg aliquots were mixed with Laemelli buffer, heat denatured for 3 min, separated by SDS-PAGE, and transferred to nitrocellulose. Non-specific binding was blocked by overnight incubation with 2% powdered milk in tris-buffered saline with 0.05% Tween-20 (Sigma Chemical Co.). Immobilized proteins were probed using antibodies specific for total MAPK proteins, the membranes were then stripped and reprobed for the phosphorylated form of the MAPK protein. Specifically, p38/pp38, pJNK/ppJNK, and pErk/ppErk (Cell Signaling Technologies, Beverly, Massachusettes). Protein bands were visualized by ECL (Amersham-Pharmacia Biotech, Piscataway, New Jersey).

C3 exotransferase treatment

Active C3 exoenzyme was introduced into the HME, HME-β-gal, HME-RhoC, and SUM149 cells using a method based on liposome encapsulation and membrane fusion, which we have termed lipoporation [16]. Briefly, cells were grown in 6-well plates until reaching a confluence of 40–50% and the medium replaced with fresh medium. Three micrograms of human recombinant C3 exotransferase (Cytoskeleton Inc., Denver, Colorado) was combine with FuGene^{TM} 6 transfection reagent (Roche-Boehringer Mannheim) and added to the cultures. As controls either an equal quantity of human recombinant tubulin or FuGene^{TM} 6 alone were added to cell cultures. The cells were incubated for 2 days at 37 °C, at which time cell-conditioned medium was harvested. Presence of the intracellular C3 exoenzyme was confirmed by visualizing the rhodamine-tagged protein using fluorescent microscopy. The efficiency and activity of both the transfected and lipoporated C3 exoenzyme were confirmed by a quantitative ADP-ribosylation assay [38].

The efficiency of *in vivo* ADP-ribosylation of RhoC GTPase by C3 exotransferase was determined as previously described [16]. Active C3 exotransferase was efficiently introduced into HME-β-gal, HME-RhoC, and SUM149, as described above. Cells were collected 48 h later, washed

in medium, and pelleted. The cells were lysed in 20 mM HEPES pH 8.0 (Sigma Chemical Co.) by 3 repeated freeze/thaw cycles. Cell lysates (10 μg) were combined with 50 ng/ml C3 exotransferase and 5×10^6 cpm (with a specific activity of 1×10^6 cpm/μl) [^{32}P]NAD (Amersham) in ADP-ribosylation buffer (20 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 1 mM AMP and thymadine, Sigma Chemical Co.), and incubated for 30 min at 37 °C. TCA-percipitable material was then recovered and radioactivity was counted on a Packard scintillation counter.

Growth assays

Monolayer culture growth rate was determined as previously described [39] by conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Chemical Co.) to a water insoluble formazon by viable cells. Three thousand cells in 200 μl medium were plated in 96-well plates and grown under normal conditions. Cultures were assayed at 0, 1, 2, 3, 5 and 7 days by the addition of 40 μl 5 mg/ml MTT and incubating for 1 h at 37 °C. The time points of the assay were chosen to sufficiently discern any effect that the inhibitors may have on cell viability, which may affect the outcomes of the phenotypic experiments. The MTT containing medium was aspirated and 100 μl DMSO (Sigma Chemical Co.) added to lyse the cells and solublize the formazon. Absorbance values of the lysates were determined on a Dynatech MR 5000 microplate reader at 540 nm.

For anchorage independent growth assays, a 2% stock of sterile low-melt agarose was diluted 1:1 with 2× MEM. Further dilution to 0.6% agarose was made using 10% FBS supplemented Ham's F-12 medium complete with growth factors, and 1 ml was added to each well of a six-well plate as a base-layer. The cell layer was then prepared by diluting agarose to 0.3% and 0.6% with 10^3 cells in 2.5% FBS supplemented Ham's F-12/1.5 ml/well. Colonies greater than or equal to 100 μ in diameter were counted after a 3-week incubation at 37 °C in a 10% CO_2 incubator.

Random motility assay

Random motility was determined using a gold-colloid assay [40]. Gold-colloid (Sigma Chemical Co.) was layered onto glass coverslips and placed into 6-well plates. Cells were plated onto the coverslips and allowed to adhere for 1 h at 37 °C in a CO_2 incubator (12 500 cells/3 ml in serum-free medium). To stimulate the cells, the serum-free medium was replaced with 5% FBS containing Ham's F-12 supplemented with growth factors and allowed to incubate for 3 h at 37 °C. The medium was aspirated and the cells fixed using 2% gluteraldehyde (Sigma Chemical Co.). The coverslips were then mounted onto glass microscope slides and areas of clearing in the gold-colloid corresponding to phagokinetic cell tracks counted.

Invasion assay

The invasion assay was performed as previously described with minor modification [39]. A 10 μl aliquot of 10 mg/ml Matrigel (Becton Dickenson, Bedford, Massachusetts) was spread onto a 6.5 mm Transwell filter with 8 μm pores (Costar, Corning, New York) and air dried in a laminar flow hood. Once dried, the filters were reconstituted with a few drops of serum-free medium. The lower chamber of the Transwell was filled with either serum-free or serum containing media. Cells were harvested and resuspended in serum-free medium with 0.1% BSA at a concentration of 3.75×10^5 cells/ml and 0.5 ml was added to the top chamber. The chambers were incubated for 24 h at 37 °C in a 10% CO_2 incubator. The cell suspension was aspirated and excess Matrigel removed from the filter using a cotton swab. The filters were then cut away from the Transwell assembly and fixed with methanol, gel side down, to a glass microscope slide. The fixed filters were stained with hematoxylin and eosin, and the cells on the entire filter were counted at a 40×-magnification individually by two investiagtors. These cells were assumed to have invaded through the Matrigel and filter. The number of cells that had invaded in the serum-free containing lower chambers was considered background and this number was subtracted from the number of cells that had invaded in response to the serum-containing medium.

Quantitation of vascular endothelial growth factor

Levels of soluble cytokines and chemokines were determined from cell-conditioned media. Cells were incubated in normal growth medium for four days. The cell-conditioned media was harvested, centrifuged for 5 min at 2,500 rpm and divided into 1 ml aliquots. The Quantikine human vascular endothelial growth factor (hVEGF; R&D Systems, Minneapolis, Minnesota) were used to measure protein levels of the 165 amino acid species of hVEGF. The enzyme linked immunoabsorbant assay (ELISA) was performed per the manufacturers recommendations.

Results

C3 exotransferase inhibition of RhoC GTPase

In a previous study we demonstrated that inhibition of RhoC GTPase activity by C3 exotransferase treatment led to decreased production of angiogenic factors [16, 17]. In order to demonstrate that the other phenotypic changes seen in the HME-RhoC transfectants are indeed due to RhoC expression, we treated the cells with C3 exotransferase. C3 exotransferase is not a specific inhibitor of RhoC *per se*, but a specific inhibitor of Rho proteins (reviewed in [41]). C3 has been demonstrated to have an affinity for RhoC and affects the formation of actin filaments *in vivo* [42]. Given that the untransfected HME, the HME-β-galactosidase control transfectants, and the HME-RhoC transfected cells were all derived from the same culture, they are likely to share the same distribution of Rho proteins, except for RhoC. Therefore, main changes of phenotype produced by C3 treatment would be ascribed to C3 induced changes in RhoC GTPase activity.

Table 1. Comparison of monolayer population doubling time and anchorage independent growth of untreated and C3 treated HME. HME transfectants and SUM149 IBC cell lines. Despite treatment of the cells with C3, monolayer population doubling time was not affected. In contrast, the ability of the RhoC expressing HME and SUM149 cells to grow under anchorage independent conditions was significantly reduced (* $P = 0.01$, ** $P = 0.001$).

	Population doubling time (hours)		Anchorage independent growth (number of cholonies)	
	Untreated	C3 Treated	Untreated	C3 Treated
HME	34 h	36 h	0 ± 0	0 ± 0.1
HME-β-gal	35 h	34 h	5 ± 0.8	17 ± 7.5
HME-RhoC	33 h	36 h	102 ± 5.4	40 ± 13.6**
SUM149	39 h	39 h	75 ± 4.9	47 ± 3.3*

Active C3 exotransferase was introduced into the cells using a liposome mediated method termed lipoporation [16]. As shown in Table 1, the population doubling time of all the cell lines tested was not significantly affected by C3 treatment. However, the ability of the HME-RhoC transfectants and the SUM149 IBC cell line to grow under anchorage independent conditions, a hallmark of malignant transformation, was significantly reduced. In contrast, C3 treatment of the HME untransfected or the HME-β-gal control did not result in any changes in their ability to grow in soft agar. The monolayer growth rate was not influenced by transfection or RhoC expression, as the HME-RhoC transfectants did not differ from the untransfected or control transfected HME counterparts, or by C3 treatment so, these data suggest that RhoC confers the ability to HME-RhoC cells to grow under anchorage independent conditions.

As demonstrated in Figure 1A, C3 treatment significantly reduced HME-RhoC and SUM149 IBC motility in a random colloidal gold assay. The HME-β-galactosidase control transfectants were unaffected by C3 treatment. Similarly, the ability of the HME-RhoC and SUM149 cells to invade a Matrigel coated filter in response to a chemoattractant was significantly reduced after C3 treatment (Figure 1B).

The activity of the C3 exotransferase was confirmed by measuring the efficiency of *in vivo* ADP-ribosylation. As shown in Figure 1C, in comparison with their non-C3 treated counterparts, all the C3 treated cell lines had a significant reduction in the levels of available sites that could be ADP-ribosylated in the *in vitro* assay. Specifically, the C3-treated HME-RhoC and SUM149 cells had a 2-fold decrease in the number of ADP-ribosylated sites compared to the non-transfected controls. These data indicate that at least half of the Rho proteins have been ADP-ribosylated *in vivo*, and therefore inhibited by C3 exotransferase. Taken together, these data demonstrate that expression and activity of RhoC GTPase is responsible for conferring the ability to grow under anchorage independent conditions, and the production of a motile and invasive cell.

Inhibition of anchorage independent growth by the LY294002 PI3K inhibitor

To determine whether the PI3K or the MAPK pathways were involved in RhoC signaling, the cells were treated with either LY294002 (a potent PI3K inhibitor) or PD98059 (a general MAPK inhibitor that blocks all arms of the MAPK pathway). To avoid confounding effects due to direct cytotoxicity, we chose concentrations of the inhibitors that inhibited signal transduction but were not cytotoxic. The cells were treated 48 h prior to plating in 0.6% soft agar and fresh medium containing each of the inhibitors was layered onto the soft agar daily. The MCF10AT c1 cell line, with a constituitvely active Ras was used as a positive control [43]. The ability of the HME-RhoC and SUM149 cells to form colonies in 0.6% soft agar was significantly reduced by treatment with the PI3K inhibitor (Figure 2). In contrast, treatment with the general MAPK inhibitor PD98059 had little effect on the colony number. The reduction in colony formation was not due to a significant change in the population doubling time of the cells treated with LY294002, as determined by an MTT monolayer growth assay performed on cells treated long-term with the inhibitors (data not shown). These data indicate the PI3K pathway, and not the MAPK pathway is involved in RhoC conferring the ability of the cells to survive and form colonies under anchorage independent conditions.

MAPK status in cell lines after inhibitor treatment

In order to determine which arms of the MAPK pathway were involved in the different aspects of the RhoC-induced phenotype, the cells were treated with a variety of MAPK inhibitors that affect different points of the pathway. The general MAPK inhibitor PD98059 effects the MAPK pathway at 2 distinct, points; (1) MEKK-1 (which activates p38 and MEK1 & 2, and therefore ERK1 & 2), and (2) directly at MEK1 & 2. The inhibitor U0126 specifically inhibits MEK1 & 2 activation. The inhibitors SKF86002 and SB22025 are inhibitors of p38 activation and of p38 itself, respectively. As demonstrated in Figure 3, all cell lines expressed p38, ERK (p42/p44), and JNK/SAPK. However, none of the untreated cell lines (A) expressed activated phospho-JNK/SAPK, sug-

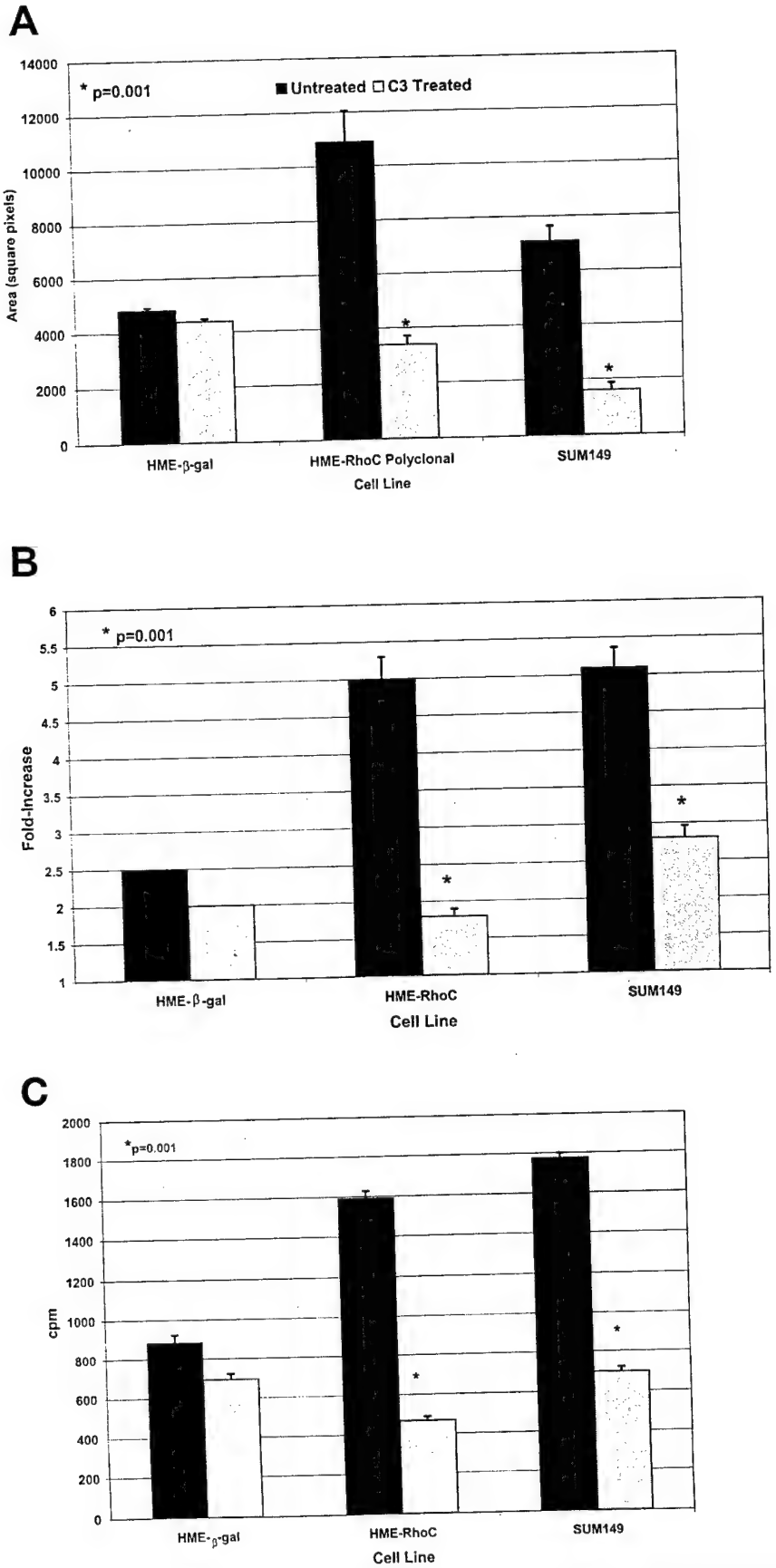

Figure 1. Comparison of the average area of migration in a colloidal gold motility assay by control HME-β-gal and RhoC overexpressing mammary cells after treatment with C3 exotransferase. Treatment of cells with C3 exoenzyme significantly reduced the motile ability of the RhoC overexpressing cells were also significantly reduced HME-RhoC and SUM149. Similarly, as demonstrated in panel B, the invasive capabilities of the RhoC overexpressing cells were also significantly reduced after C3 treatment as determined in a Matrigel invasion assay. To determine the extent of Rho inhibition by C3 exotransferase, an *in vitro* ADP-ribosylation assay was performed (panel C). The number of ADP-ribosylated targets was greatly reduced in the HME-RhoC and SUM149 cells, thus indicating that C3 exotransferase treatment had effectively blocked the Rho targets within those cells.

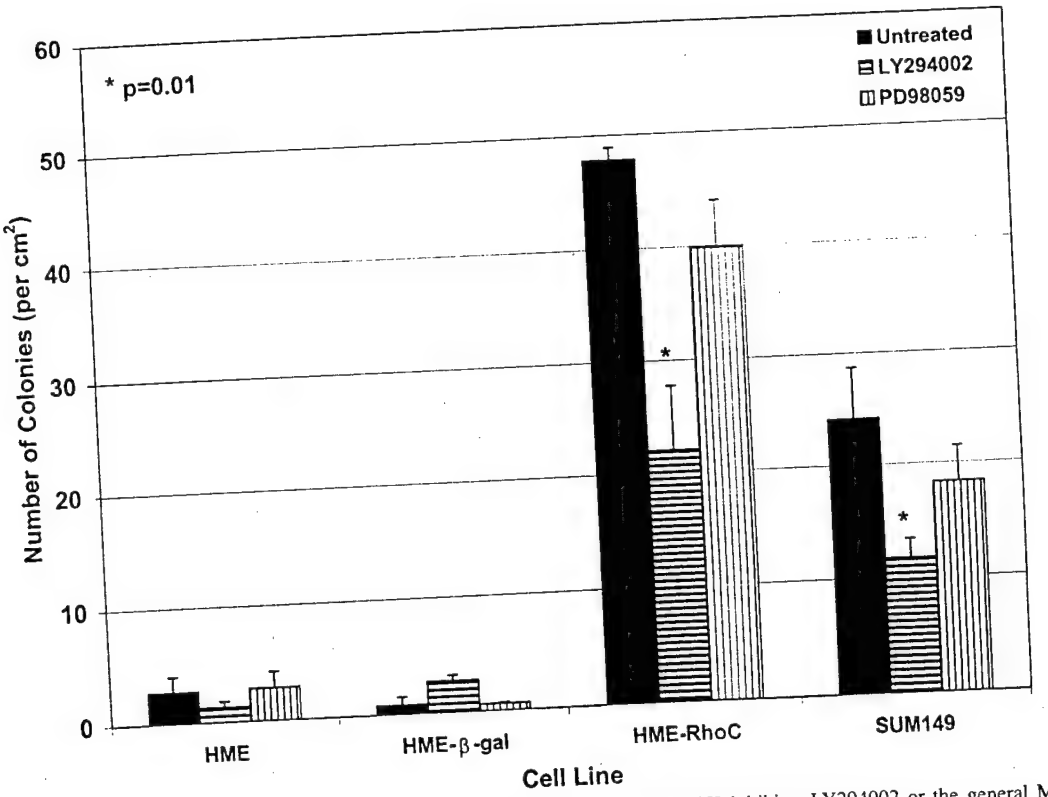

Figure 2. Anchorage independent growth in 0.6% soft agar after treatment with either the PI3K inhibitor LY294002 or the general MAPK inhibitor PD98059. The ability of the.RhoC overexpressing cells HME-RhoC and SUM149 was significantly reduced after treatment with the LY294002. but not with PD98059. These data suggests that RhoC-mediated anchorage independent growth. is signaled through the PI3K and not the MAPK pathway in these mammary cells.

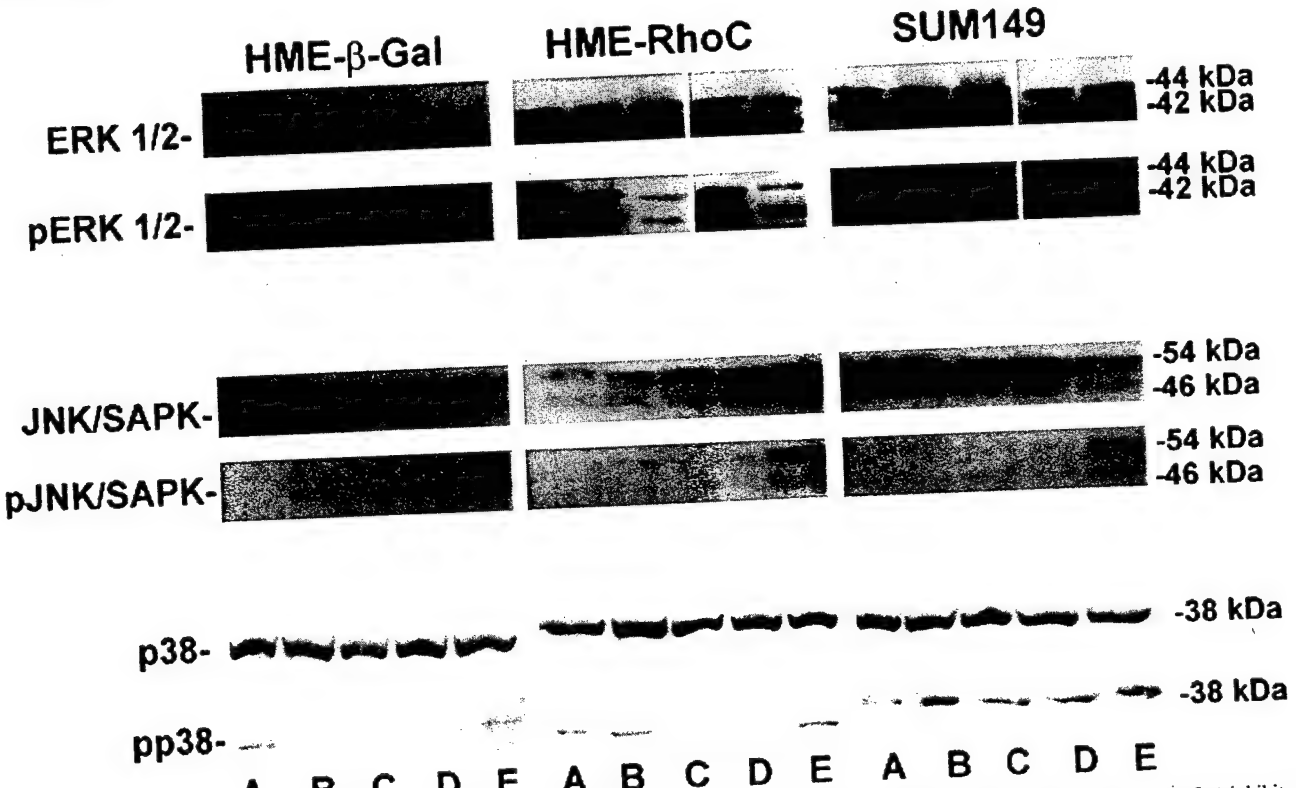

Figure 3. Western blot analysis of basal and phosphorylated (activated) levels of different arms of the MAPK pathway before (A) and after inhibitor treatment with SKF86002 (B), PD98059 (C), U0126 (D), or C3 exotransferase (E). All the cell lines tested expressed ERK (p42/p44). JNK/SAPK. and p38. However, none of the untreated cell lines (A) expressed activated phospho-JNK/SAPK. suggesting that only pp38 and phospho-ERK are involved in RhoC signal transduction. Each set of Western blots looking at p38, JNK/SAPK and ERK were performed separately on the same cell lysates. Each individual blot in the set (i.e. total protein *versus* the phosphorylated form of that protein) was the same blot stripped and re-probed.

gesting that only pp38 and phospho-ERK are involved in RhoC signal transduction. Each set of Western blots looking at p38, JNK/SAPK and ERK were performed separately on the same cell lysates. Each individual blot in the set (i.e., total protein versus the phosphorylated form of that protein) was the same blot stripped and re-probed. Each set of Western blots looking at p38, JNK/SAPK and ERK were performed separately on the same cell lysates. Each set of Western blots compares total protein *versus* the phosphorylated form of that protein of the same blot. Treatment of the cells for 24 h with SKF86002 (B), PD98059 (C), U0126 (D), or C3 exotransferase (E), led to a decrease in the levels of the active phosphorylated form of the target protein(s), while the basal levels remained unchanged. Interestingly, C3 treatment of the cells lead to increased phosphorylation of JNK/SAPK.

Effect of inhibition of MAPK on motility and invasion

Because of the postulated relationship between Rho-induced motility and Ras activation of the MAPK pathway, we set out to understand how are the MAPK signaling cascades are involved in Rho-modulated motility and invasion. To accomplish this we treated the cells with the various MAPK inhibitors described above. The cells were treated with the MAPK inhibitors 48 h prior to assessing motility and invasion. No significant decrease in population doubling time was observed for the cells treated with inhibitors, as determined over a seven-day assay (data not shown).

As demonstrated in Figure 4A, all of the MAPK inhibitors had a significant ($P = 0.01$) effect on the motility of the HME-RhoC and SUM149 cell lines. The areas of the phagokinetic tracks were reduced to nearly the level of the HME-β-gal control cell line, which was unaffected by any of the MAPK inhibitors. Since all of the MAPK inhibitors had an effect on the motility of the cells, this suggested that multiple arms of the MAPK pathway are involved in RhoC mediated motility. Motility of the MCF10AT c1 positive control cell line that has a constituatively active Ras was also affected by all four of the MAPK inhibitors, although the motility of these cells is much reduced compared to the HME-RhoC and SUM149 cells.

Next, we concentrated on the cells ability to invade through a Matrigel coated filter (Figure 4B). The invasive capabilities of the cells are described as fold-increase in invasion over untransfected HME controls. Treatment with all four of the MAPK inhibitors reduced the invasive capabilities of the HME-RhoC and SUM149 cell lines. The HME-β-gal control cells were not significantly affected, by the other MAPK inhibitors. The invasive capabilities of the MCF10AT c1 cells were the same as the HME-β-gal control cells, and were likewise unaffected by the MAPK inhibitors. When cells were treated with a combination of the LY294002 and PD98059 inhibitors, the level of inhibition was similar to that of the PD98059 inhibitor alone (data not shown), suggesting that the PI3K pathway is not involved in either motility or invasion.

Taken together, these data suggest that RhoC induced motility and invasion is mediated to a significant extent by the p38 and ERK arms of the MAPK pathway. This is shared with Ras alone induced motility, but active Ras is not sufficient to produce an invasive phenotype in the MCF10A cells. In all these experiments the concentrations of inhibitors used did not effect cell doubling times or cell viability.

VEGF production after inhibition of the MAPK pathway

In a previous study, we demonstrated that RhoC overexpression leads to increased production of angiogenic factors, particularly vascular endothelial growth factor (VEGF) [16, 17]. Since VEGF production was significantly increased in RhoC expressing breast cells, and considering its importance in as an angiogenic factor, VEGF expression was the logical endpoint to study after inhibitor treatment. Treatment of the mammary cells with the different MAPK inhibitors resulted in decreased VEGF production by the HME-RhoC and SUM149 IBC cell lines (Figure 5). The greatest reduction in VEGF production was seen when the cells were treated with the inhibitor SB22025, which prevents p38 activation. Treatment with the inhibitor SKF86002, an inhibitor of phosho-p38 activity, resulted in the second greatest decrease in VEGF production. Taken together, these data suggest that activation of the p38 arm of the MAPK pathway is responsible for production of VEGF simultaneously by RhoC overexpression.

Discussion

The highly invasive and metastatic phenotype of IBC is one of the hallmarks of its unique clinical manifestations and the major cause of the poor outcome of many patients who are diagnosed with IBC. In a previous set of studies, our laboratory identified RhoC GTPase to be overexpressed in IBC and responsible for anchorage independent growth, cellular motility and invasion, and production of angiogenic factors.

In the current study, we begin to delineate the signaling pathways responsible for each aspect of the RhoC-mediated phenotype. We hypothesized that for RhoC GTPase to achieve diverse phenotypic attributes, cell signaling must take place through several signal transduction pathways. We utilized specific inhibitors of different points of the PI3K and MAPK pathways, an approach which has proven successful in similar previous studies [44, 45].

The MAPK pathway has been previously implicated in Ras and RhoA signaling, while the JNK/SAPK pathway has been shown to be mediated by Rac1 and cdc42 signaling, and all have been shown to use the PI3K pathway (reviewed in [46, 47]). These pathways, depending on cell type, have been attributed to participate in growth/survival and motility (reviewed in [29]). However, the signal transduction pathway(s) utilized by RhoC during growth, motility and invasion has not been described in any cell type. In the current study we examined RhoC signal transduction in the SUM149 IBC and HME-RhoC breast cell lines. Because RhoC appears to be a major determinant of a clinically well-defined mammary cancer metastatic phenotype it is es-

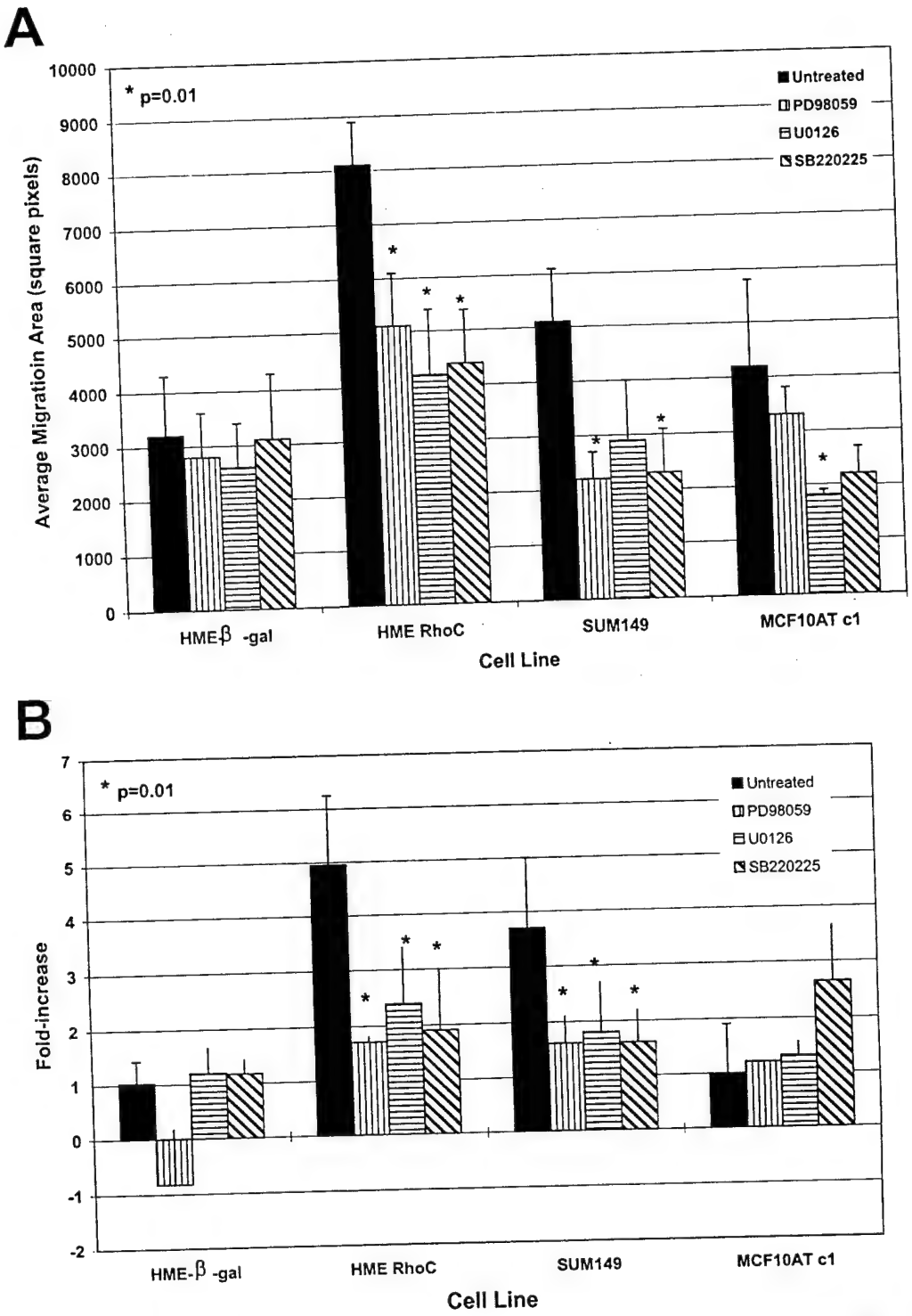

Figure 4. Effects on motility and invasion of RhoC overexpressing cells after treatment with PD98059, U0126 or the p38 inhibitor SB220225. Panel A demonstrates a significant decrease in the motility of the RhoC overexpressing HME-RhoC and SUM149 cells treated with the various MAPK inhibitors. Similarly, the ability of these cells to invade through a Matrigel coated filter was also significantly reduced after treatment with the MAPK inhibitors (panel B). These data suggest that RhoC mediated motility and invasion is mediated through the MAPK pathway, to a large extent through activated p38.

pecially relevant to understand how RhoC elicits multiple actions in breast tissue.

Using MAPK and PI3K inhibitors at concentrations below cytotoxic and cytostatic levels, we have determined that the PI3K pathway is involved in the ability of RhoC overexpressing cells to grow under anchorage independent conditions without effecting monolayer growth. We also determined that signaling through the MAPK pathway is involved in motility, invasion and the production of angiogenic factors. Specifically, we found that the ERK and p38 arms of the MAPK signaling complex are involved in motility and invasion, as no one inhibitor of the individual arms com-

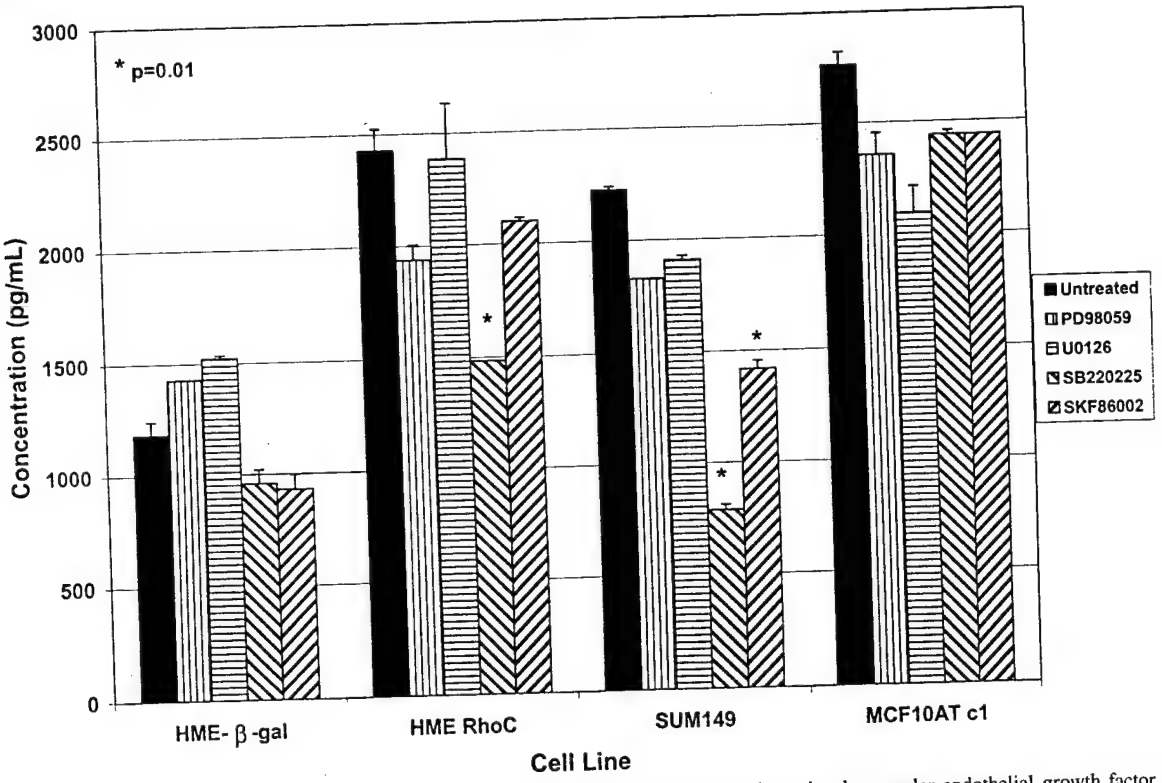

Figure 5. The effect of MAPK inhibitors on the production and secretion of the pro-angiogenic molecule vascular endothelial growth factor (VEGF). Production of VEGF by the HME-RhoC and SUM149 cell lines were significantly reduced when the cells were treated with the p38 inhibitors SB220225 or SKF86002. Taken together, these data suggest that activation of the p38 arm of the MAPK pathway is responsible for production of VEGF simultaneously by RhoC overexpression.

pletely blocked motility of the HME-RhoC or SUM149 IBC cell lines. In addition, although it is expressed, we know that JNK/SAPK does not appear to be involved given that it is not phosphorylated or active in any of the breast lines (IBC or HME) studied.

Previous experiments have demonstrated that the various signal transduction pathways have diverse effects in different cell types activated by a variety of stimuli. For example in Schwann cells, PI3K activation by Rac1 leading to lamellipodia formation and motility has been shown to occur upon stimulation by insulin-like growth factor-I [25]. Whereas stimulation of adipocytes with insulin leads activation of the PI3K pathway and Rho-mediated glucose uptake [48]. In support of our data, Amundadottir and Leder [49] demonstrated that regardless of the oncogene involved in transformation, the PI3K pathway was involved in conferring anchorage independent growth to transformed mammary epithelial cells. They also demonstrated that anchorage independent growth of mammary cells transformed by Her2/neu, v-Ha-ras, and c-myc, could not be inhibited by treatment with the MAPK inhibitor PD98059. Thus consistent with our study of RhoC-expressing cells, it appears that the PI3K pathway is exclusively involved in conferring anchorage independent growth, without involving the MAPK pathway.

Several studies have demonstrated that activation of the MAPK pathway can lead to cell migration and invasion of fibroblasts, keratinocytes and endothelial cells [50–52]. Further, it has been well documented that the Rho proteins can activate the MAPK cascade stimulating various aspects of cellular motility [29, 53–55]. Rac1 and cdc42 have been shown to signal gene transcription through JNK/SAPK and RhoA through p38, or when bound to fibronectin, ERK 1 & 2 [22, 53, 56, 57]. Akin to RhoA, we have made similar observations for RhoC, having demonstrated activation of both p38 and ERK, but not JNK/SAPK, in IBC and transfected HME cells. During motility, a dynamic interplay between Rac1, cdc42 and Rho must occur to form lamellipodia, fillipodia, focal adhesions, and stress fibers [7, 58, 59]. 'Cross-talk' between these molecules results in reciprocal activation of Rho with Rac1 and cdc42 [59–61]. Therefore, each arm of the MAPK pathway may be involved in motility and invasion during some point of the process.

In a previous study we demonstrated that levels of VEGF was significantly elevated due to RhoC overexpression [16]. In support, other laboratories have demonstrated that expression of angiogenic factors is mediated by the p38 MAPK cascade [26–28]. An increasing body of evidence suggests that the mode of VEGF induction (i.e., MAPK vs. PI3K) by activated H-*ras* is a cell-type specific process, with cells of epithelial origin signaling more commonly through the MAPK pathway and those cells of mesodermal origin utilizing the PI3K pathway [62]. Stimulation of a variety of breast cancer cell lines with heregulin results in activation of p38 and subsequent upregulation of VEGF expression and secretion [28]. Similarly, epidermal growth factor stimulation of squamous cell carcinoma cell lines, results in activation of both p38 and ERK, which in turn, leads to expression

of fibroblast growth factor-binding protein (FGF-BP), a potent angiogenic modulator [26]. Furthermore, it has been suggested that Rho proteins and the p38-MAP kinase pathway modulate IL-8 expression [63, 64]. IL-8 expression has profound biological consequences: it is a potent angiogenic, mitogenic and chemotactic factor in several malignancies including breast and prostate cancer [65–69]. Still others have suggested that FGF2, acting in an autocrine and paracrine fashion, can induce IL-6 expression through p38. In future studies we will determine whether IL-6, IL-8, and FGF2 production is also modulated by the p38 pathway in RhoC overexpressing mammary cells.

In conclusion, we have begun to identify the different signal transduction pathways involved in RhoC GTPase driven phenotypes associated with highly metastatic inflammatory breast cancer. We specifically demonstrated that anchorage independent growth is mediated via the PI3K pathway. Induction of motility and invasion are mediated through activation of the ERK and p38 arms of the MAPK pathway, and the production of VEGF is mediated primarily by p38 activation. This study provides new insight into the signal transduction pathways of an aggressive disease mediated by overexpression and activation of RhoC GTPase and suggests new potential targets for therapeutic interventions focused on the biological actions of RhoC.

Acknowledgements

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WISP3 is a novel tumor suppressor gene of inflammatory breast cancer

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Inflammatory breast cancer (IBC) is an aggressive form of breast cancer with a 5-year disease-free survival of less than 45%. Little is known about the genetic alterations that result in IBC. In our previous work, we found that WISP3 was specifically lost in human IBC tumors when compared to stage-matched, non-IBC tumors. We hypothesize that WISP3 has tumor suppressor function in the breast and that it may be a key genetic alteration that contributes to the unique IBC phenotype. The full-length WISP3 cDNA was sequenced and cloned into an expression vector. The resulting construct was introduced in to the SUM149 cell line that was derived from a patient with IBC and lacks WISP3 expression. In soft agar, stable WISP3 transfectants formed significantly fewer colonies than the controls. Stable WISP3 transfectants lost their ability to invade and had reduced angiogenic potential. WISP3 transfection was effective in suppressing *in vivo* tumor growth in nude mice. Mice bearing WISP3 expressing tumors had a significantly longer survival than those with vector-control transfectant tumors. Our data demonstrate that WISP3 acts as a tumor suppressor gene in the breast. Loss of WISP3 expression contributes to the phenotype of IBC by regulating tumor cell growth, invasion and angiogenesis.

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Keywords: WISP3; breast cancer; tumor suppressor gene; angiogenesis

Introduction

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer and accounts for approximately 6% of new breast cancer cases annually in the United States (Jaiyesimi *et al.*, 1992; Lee, 1924). It is also a very distinct clinical and pathological form of breast carcinoma. It is characterized clinically by erythema, skin nodules, dimpling of the skin (termed 'peau d'orange') and by a rapid onset of disease, typically progressing within 6 months (Jaiyesimi *et al.*, 1992; Lee, 1924; Merajver *et al.*, 1997; Swain *et al.*, 1987). Pathologically, carcinomatous tumor emboli spread through the dermal lymphatics which are responsible for the clinical signs and symptoms (Lee, 1924). IBC is generally not associated with precursor lesions (Jaiyesimi *et al.*, 1992; Lee, 1924). From the outset, IBC is highly invasive and it appears to be capable of metastases from its inception. It is estimated that nearly all women with IBC have nodal involvement at the time of diagnosis, and over one-third have distant metastases (Merajver *et al.*, 1997; Swain *et al.*, 1987). In spite of new advances in breast cancer therapy, the overall 5-year disease-free survival rate is less than 45% (Merajver *et al.*, 1997; Swain *et al.*, 1987).

The genetic alterations underlying the development and progression of IBC were unknown until recently. Our laboratory identified two genes that are consistently and concordantly altered in human IBC when compared to stage-matched, non-IBC tumors: loss of WISP3 and over-expression of RhoC-GTPase (van Golen *et al.*, 1999).

WISP3 has been identified as a member of the CCN family of proteins, which have important biological functions in normal physiology as well as in carcinogenesis (Perbal, 2001). There are only two publications in the literature on the biological effects of WISP3 and very little is known about the expression pattern and function of this novel gene (Hurvitz *et al.*, 1999; Pennica *et al.*, 1998). We hypothesize that WISP3 may function as a growth-suppressing factor in breast carcinogenesis and that it may be a key genetic alteration in IBC in particular. To test this hypothesis, we set out to determine whether restoring WISP3 expression in an IBC cell line, SUM149, could abrogate or modulate the highly malignant IBC phenotype. We observed that stably transfected SUM149/WISP3 cells exhibited a striking decrease in proliferation rate, anchorage independent growth, invasion, and tumor growth in nude mice compared to empty vector transfectants. This work suggests new insights into the role of WISP3 in carcinogenesis in general.

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Results

Transfection with the SUM149 cell line with WISP3 cDNA

Figure 1 shows the Western blot analysis of WISP3 protein in whole cell lysates from four clones of SUM149/WISP3 stable transfectants, SUM149/Flag (transfection controls), SUM149 wild type, and HME cells (human mammary epithelial cells). We used a specific anti-WISP3 antibody (gift from Dr Mathew Warman).

Statistically significant differences were found when each SUM149/WISP3 clone was compared to the SUM149/Flag control ($P<0.04$, t-test).

WISP3 induces a striking morphologic change in IBC cell line

After stable transfection with WISP3 cDNA, we observed marked morphologic differences between the SUM149/WISP3 clones and SUM149/Flag control (Figure 2a). Whereas the control SUM149/Flag cells had a characteristic tightly packed, cobblestone appearance, with a cuboidal shape and high nuclear to cytoplasmic ratio, the SUM149/WISP3 transfectants were heterogeneous in size and morphology. They became large, round, and flat with abundant granular and vacuolated cytoplasm and ill-defined cell borders. A detailed ultrastructural analysis by electron microscopy demonstrate that the WISP3 transfectants exhibited numerous cytoplasmic lysosomal bodies surrounded by a membrane, some containing electron-dense material and invaginated and convoluted nuclei (Figure 2b). These morphologic features have been described in cells undergoing senescence (Romanov *et al*., 2001; Tsugu *et al*., 2000).

WISP3 partially abrogates angiogenesis in IBC

As illustrated in Figure 3a, SUM149/WISP3 clones produce significantly decreased levels of FGF2 (bFGF) and VEGF, key pro-angiogenic factors. The levels of IL-6, another pro-angiogenic cytokine were significantly decreased. To investigate whether the decreased production of angiogenic factors induced by restoration of WISP3 expression resulted in the inhibition of neovascularization, we carried out rat aortic ring assays. After 10 days of incubating a rat aortic ring with a 10× concentration of the cell culture supernatant, fewer vessels were formed by SUM149/WISP3 cells when compared to the controls (Figure 3b). The SUM149/WISP3 transfectants formed a mean number of 26.33 new vessels (s.e.m. = 1.63), and the SUM149/Flag control cells formed a mean number of 69.33 new vessels (s.e.m. = 4.15) (t-test, $P=0.0003$). These results show that expression of WISP3 depressed the strength of the tumor-induced signal for angiogenesis.

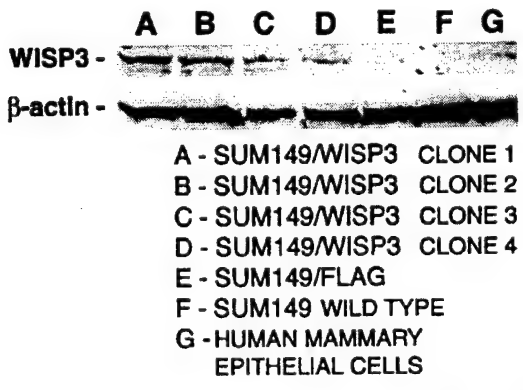

Figure 1 Western blot using a specific anti-WISP3 antibody demonstrates successful transfection and expression of WISP3 in SUM149 cells (lanes A–D). Lane E: transfection control (SUM149/Flag). Lane F: wild-type SUM149. Lane G: HME cells with normal expression of WISP3

Expression of WISP3 reduces the proliferation rate, anchorage-independent growth, and the invasive ability of IBC cells

As anchorage independent growth is a hallmark of malignant transformation, we investigated whether expression of WISP3 in highly malignant SUM149 cell line abolished this feature. After 14 days of growth in soft agar four WISP3 expressing clones formed significantly fewer and smaller colonies than the control cells ($P<0.05$), (Figure 4a,b). Figure 4c shows the results of the MTT assay and illustrates that the

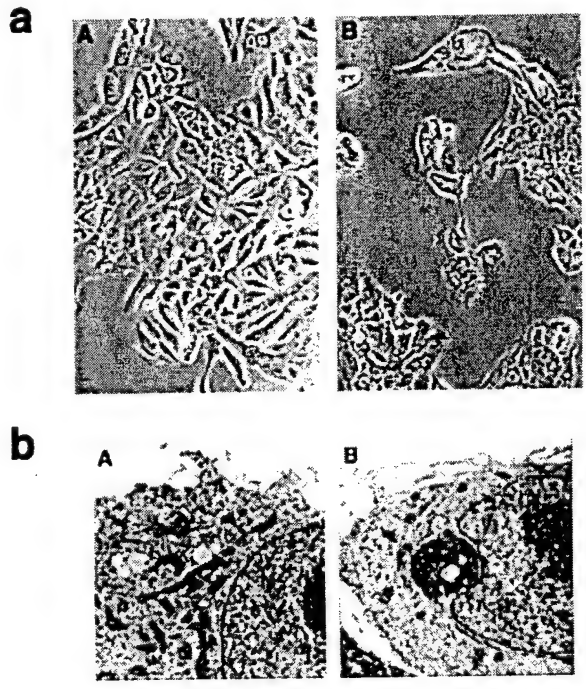

Figure 2 (**a**) Phase contrast microscopy showing the vacuolization and morphologic changes induced by restoring WISP3 expression in SUM149 cells. A: SUM149/Flag (controls). B: SUM149/WISP3 (X 200). (**b**) Electron microscopy. A: SUM149/Flag, and B: SUM149/WISP3 transfected cell showing a prominent intracytoplasmic dense vacuole

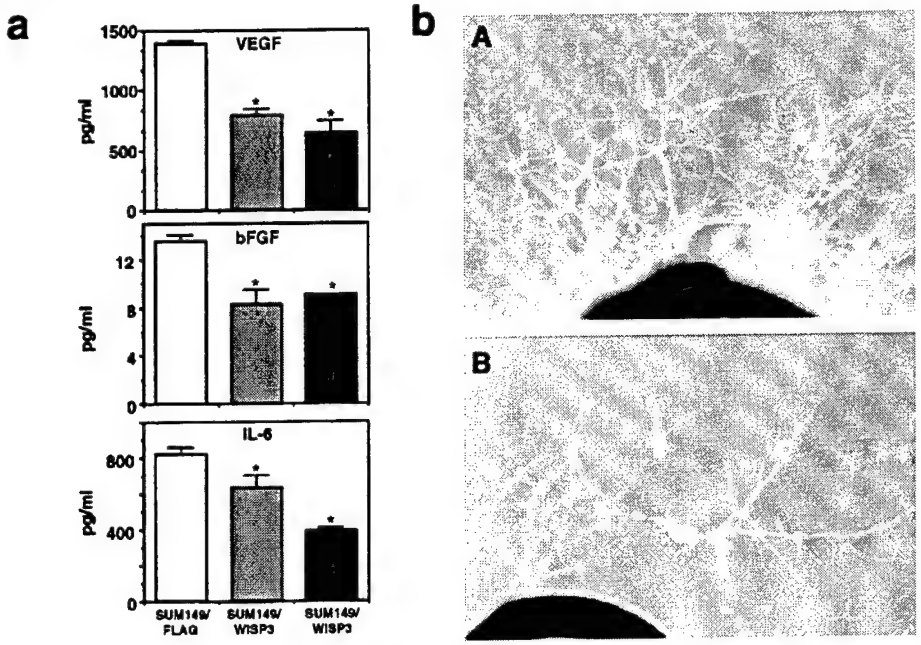

Figure 3 (**a**) Decrease in key angiogenic factors measured by ELISA, as a result of restoration of WISP3 expression in SUM149 cells. Results are expressed as mean ± s.d. $*P<0.05$. (**b**) Rat aortic ring assay showing marked decreased in new vessel formation from the pre-existing aortic ring bathed in conditioned media from SUM149/WISP3 (B), and SUM149/Flag controls (A). (100×)

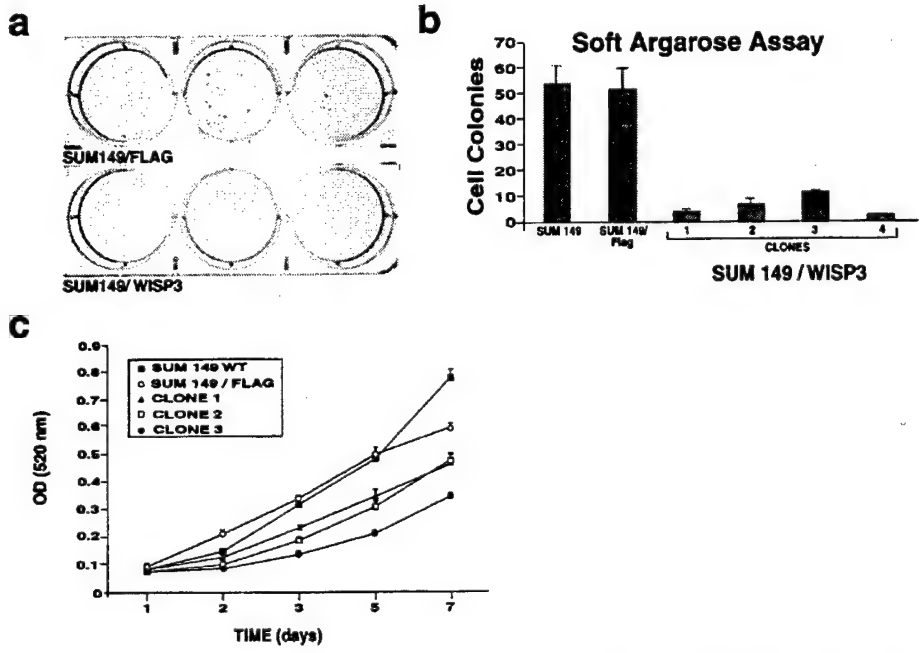

Figure 4 (**a**) and (**b**) Anchorage-independent growth in soft agar. Restoration of WISP3 in SUM149 cell line greatly decreased the number of the colonies formed. (**c**) Effect of stable WISP3 transfection on the proliferation of SUM149 cells studied with the MTT assay. Results are expressed as mean ± s.d. of three independent experiments. Three thousand cells were assessed in each plate ($P<0.05$)

proliferation rate of three different clones of SUM149 cells transfected with WISP3 decreased significantly when compared to controls ($P<0.05$). The ability of the transfectants to invade and intravasate was studied using the sea urchin extracellular membrane (SU-ECM) invasion assay. This assay provides a naturally serum-free, selectively permeable basement membrane that closely mimics the type of extracellular matrix that

cells encounter *in vivo*. Mean invasion percentages were calculated from three independent analyses of 50–100 random cells. We found that transfection with WISP3 cDNA completely abolished the ability of IBC cells to invade, compared to the control (the mean $\pm$ s.d. for invasion of SUM149/flag cells was 14 ± 3, when compared to 0 for both clones of SUM149/WISP3).

WISP3 expression inhibits cellular growth by inducing accumulation of $p27^{kip1}$ and $p21^{waf1}$ in cancer cells and induces apoptosis

Based on the morphologic changes induced by transfection with WISP3, we set out to investigate whether WISP3 caused alterations in the regulation of the cell cycle, that could in turn be contributing to the inhibition of cellular proliferation, anchorage-independent growth, and *in vivo* tumor growth. To elucidate the possible effect of WISP3 on the cell cycle regulators, the round and vacuolated cells (SUM149/WISP3) were assayed for the presence of several key cell cycle regulators by Western immunoblots. Interestingly, when compared to the control cells, the level of $p27^{kip1}$ and $p21^{waf1}$ were increased in the WISP3 transfectants when compared top SUM149/flag control cells ($P=0.0003$ and $P=0.003$ for $p27^{kip1}$ expression for clones 1 and 2 respectively, and $P=0.001$ and $P=0.06$ for $p21^{waf1}$ expression for clones 1 and 2 respectively, *t*-test, Figure 5). Concordantly, SUM149/WISP3 cells exhibited decreased levels of cyclin E and PCNA, a reliable marker for cellular proliferation, when compared to the SUM149/flag control cells ($P=0.02$ and $P=0.02$ for cyclin E expression for clones 1 and 2 respectively, and $P=0.0003$ and $P=0.003$ for PCNA expression for clones 1 and 2 respectively, *t*-test, Figure 5).

We noticed an increased percentage of apoptotic cells after stable transfection with WISP3 detected by the Annexin V assay by flow cytometry. SUM149/WISP3 transfectants had 30% apoptotic cells when compared to SUM149/Flag control cell line with only 13% apoptotic cells.

WISP3 inhibits in vivo *tumor growth and improves survival of mice*

Given that WISP3 affects cell cycle regulation, proliferation rates and anchorage independent growth, we investigated the effect of restoration of WISP3 expression on SUM149 xenograft tumor growth and survival of the host mice. Three groups of 10–11 mice were injected with two clones of SUM149/WISP3 stable transfectants and a control group (SUM149/Flag and SUM149 wild-type, five mice each). Mice with orthotopically implanted tumor cells were monitored weekly for tumor formation over an 8-week period. By the 8th and final week of the experiment, palpable tumors developed in 20 of the 21 mice that received injection of the SUM149/WISP3 clones and in nine out of 10 mice given injection of control clones (five injected with SUM149/Flag and five injected with wild-type SUM149). The rate of tumor formation between WISP3 transfectants and controls differed (Table 1). Specifically, the clones transfected with WISP3 formed tumors at a slower rate than the controls (logistic regression analysis adjusted for longitudinal correlation, $P=0.05$). Moreover, when tumor volume was compared at each week (starting at week 2), it was significantly lower in the tumors formed by SUM149/WISP3 transfectants than in the tumors formed by the controls ($P=0.05$). When the slopes of the regression lines were compared, striking differences were found between the WISP3 transfectants and control cells ($P<0.001$, Figure 6a,b). At week 5, two tumors from each clone and from the control were excised. WISP3 mRNA expression was confirmed by quantitative RT–PCR. Levels of WISP3 expression were found to be similar to those of preinjection. Taken together, these results demonstrate that tumors formed by SUM149/WISP3 take longer to grow and are smaller than controls.

To address whether the tumors formed by the transfectants expressing WISP3 were pathologically different from the control, histopathologic study of the tumors was carried out. As shown in Figure 6c, SUM149/Flag tumors were highly anaplastic, had

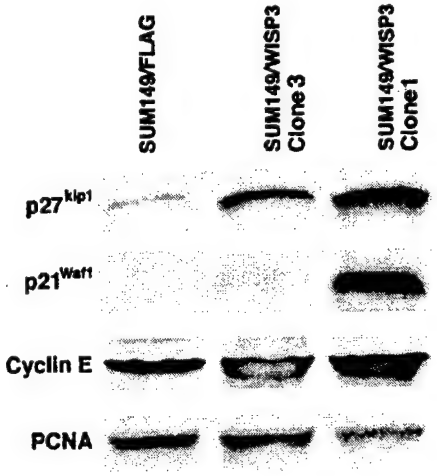

Figure 5 Western immunoblot of cell culture media of control (SUM149/FLAG), and two WISP3 expressing clones using antibodies for p27kip1, p21waf1/cip1, cyclin E, and PCNA

Table 1 Rate of tumor formation after orthotopic injection in the mammary fat pad of SUM149 wild-type, SUM149/Flag, and two stable clones of SUM149/WISP3 transfectants in female athymic nude mice. Tumors formed by the WISP transfectants took a longer time to develop than tumors formed by the control cells ($P=0.05$, logistic regression analaysis)

	2 weeks n (%)	*4 weeks* n (%)	*6 weeks* n (%)	*8 weeks* n (%)
SUM149 wild-type	5/5 (100)	5/5 (100)	5/5 (100)	5/5 (100)
SUM149/Flag	4/5 (80)	4/5 (80)	4/5 (80)	4/5 (80)
SUM149/WISP3 Clone 1	9/11 (82)	10/11 (91)	10/11 (91)	10/11 (91)
SUM149/WISP3 Clone 3	3/10 (30)	5/10 (50)	9/10 (90)	10/10 (100)

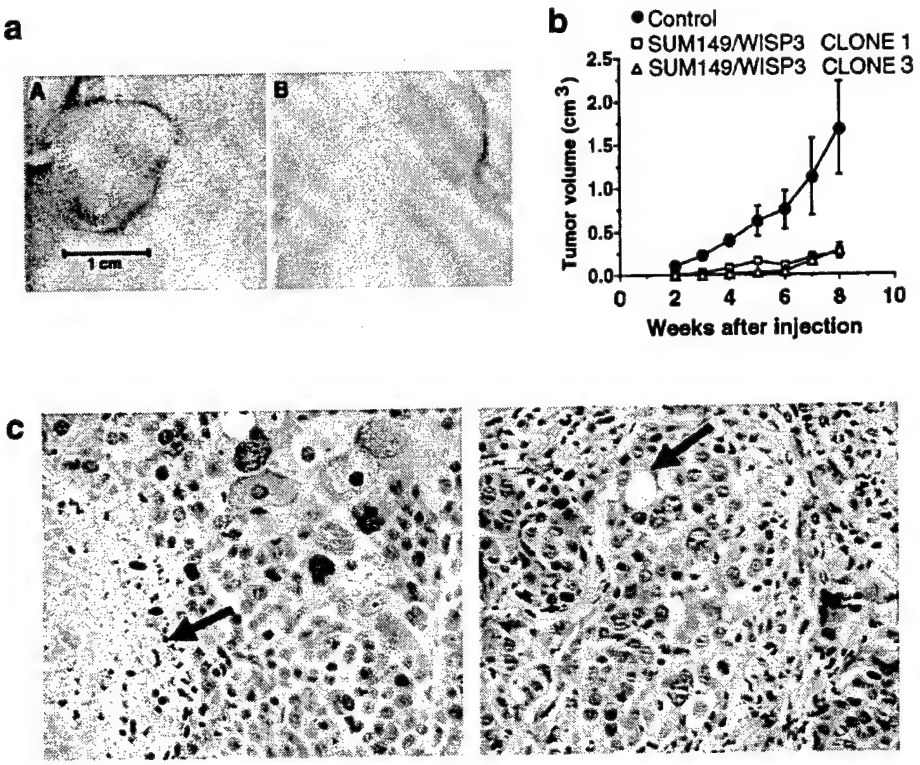

Figure 6 (**a**) SUM149 cells transfected with WISP3 (A) formed smaller tumors than SUM149/Flag controls. Pictures were taken 5 weeks after injection (B). (**b**) Effect on tumor volume of restoration of WISP3 in SUM149 cells. When the slopes of the curves were compared, a significant difference was found between the transfectants and controls ($P<0.001$). Results are expressed as mean $\pm$ s.e.m. Wild-type SUM149 were represented together with SUM149/FLAG because no statistically significant difference between the 2 groups was found. (**c**) Pathological features of the tumors. The tumors formed by SUM149/Flag controls are pleomorphic, with extensive necrosis (arrow) and mitoses. The tumors formed by the SUM149/WISP3 clones are better differentiated, the cells are smaller, there is less necrosis and mitoses. The arrow points to a glandular lumen. (Hematoxylin and eosin stain, light microscopy, $\times 400$)

strikingly atypical cells forming solid aggregates, with no glandular formation, a sign of poor differentiation, and extensive areas of necrosis. The cells exhibited high mitotic activity (mean 88 mitoses per 10 high power field ($400\times$)) and numerous abnormal mitoses. In contrast, the tumors derived from SUM149/WISP3 clones had less nuclear pleomorphism and slightly better differentiation evidenced by occasional gland formation. These tumors had less necrosis and the mitotic activity was greatly decreased (mean 11 mitoses per 10 high power fields).

Discussion

WISP3 is located in chromosome 6q21–22 and encodes for a 354 amino acid, 39 292 Da protein with the modular architecture of the CCN family of proteins which includes connective tissue growth factor (CTGF), Cyr61, Nov, WISP1 and WISP2 (Babic *et al*., 1998; Hashimoto *et al*., 1998; Hurvitz *et al*., 1999; Kireeva *et al*., 1996; O'Brien and Lau, 1992, Pennica *et al*., 1998; Perbal, 2001; Wong *et al*., 1997; Xu *et al*., 2000). The CCN proteins participate in fundamental biological processes such as cell proliferation, migration, wound healing, angiogenesis and tumorigenesis (Perbal, 2001). They have a range of biological properties that might be dependent upon the cellular context (Perbal, 2001).

In tumorigenesis, Cyr61 and WISP1 were reported to act as positive regulators of cell growth (Babic *et al*., 1998; Hashimoto *et al*., 1998; Kireeva *et al*., 1996; O'Brien and Lau, 1992; Pennica *et al*., 1998; Wong *et al*., 1997; Xu *et al*., 2000); Nov expression was found to correlate with the development of metastases in Ewing's sarcomas, prostate cancer, and renal cell carcinomas (Perbal, 2001), whereas it was found to act as a negative growth regulator of glioblastoma cell lines (Li *et al*., 1996). Furthermore, in primary human colonic adenocarcinomas, the expression of WISP2 was significantly decreased compared to normal colon (Pennica *et al*., 1998), and it was not detected in the epithelial tumor cells of mammary carcinoma obtained from Wnt-1 transgenic mice (Pennica *et al*., 1998). Similarly, in our study we could not detect WISP3 mRNA expression in 80% of the IBC human tissues studied (van Golen *et al*., 1999). The expression of ELM1/WISP1, which had been reported to suppress the metastatic potential of murine melanoma cells (Hashimoto *et al*., 1998), was significantly increased in

most human colonic carcinomas (Pennica *et al.*, 1998; Xu *et al.*, 2000). These data provide compelling evidence that the CCN proteins may have opposing functions in carcinogenesis in different cell types and tissues.

There are only two previous publications in the literature on the biologic properties of WISP3 (Hurvitz *et al.*, 1999; Pennica *et al.*, 1998). In one of these studies, the WISP3 gene was significantly over-expressed in human colon carcinoma (Pennica *et al.*, 1998) whereas Hurvitz *et al.* (1999) found loss of function mutations in the WISP3 gene to be associated with progressive pseudorheumatoid dysplasia in humans (Hurvitz *et al.*, 1999). Patients with progressive pseudorheumatoid dysplasia due to WISP3 loss-of-function mutations have not been reported to have higher rates of cancer; however this has not been systematically sought in this cohort (Hurvitz *et al.*, 1999). Our data provide evidence that WISP3 has tumor suppressor function in the mammary gland.

Using a modified version of the differential display technique and *in situ* hybridization of human breast cancer tissues, we found that WISP3 is lost in 80% of IBC and in 21% of stage matched, non IBC tumors ($P=0.0013$, Fisher's exact test) (van Golen *et al.*, 1999). WISP3 loss was found in concert with RhoC-GTPase over-expression in 90% of archival IBC patient samples, but not in stage-matched non-IBC tumors (van Golen *et al.*, 1999). Our laboratory demonstrated that RhoC-GTPase over-expression produces a motile and invasive phenotype in human mammary epithelial cells, primarily through formation of actin stress fibers and focal adhesion points (van Golen *et al.*, 2000b). We hypothesized that loss of expression of WISP3 contributes to the development of the IBC phenotype by altering angiogenesis and tumor growth, therefore complementing the role of RhoC-GTPase over-expression.

One of the features that define the IBC phenotype as unique is its rapid progression, with fast growth and invasion, making it the most lethal form of locally advanced breast cancer (Jaiyesimi *et al.*, 1992; Lee, 1924; Merajver *et al.*, 1997; Swain *et al.*, 1987). IBC is characterized by a rapid onset of disease, typically arising within 6 months, and by the time of diagnosis, the majority of patients have locoregional and distant metastatic disease (Jaiyesimi *et al.*, 1992; Lee, 1924; Merajver *et al.*, 1997; Swain *et al.*, 1987). Transfection of the IBC cell line SUM149 with WISP3 cDNA inhibited some of these key phenotypic characteristics.

The proliferation rate of highly aggressive SUM149 cell line was decreased, and growth under anchorage independent conditions was greatly reduced in the stable WISP3 transfectants. In comparison with the control cells, the WISP3 transfectants produced 6–16-fold less colonies than the control cells. Similarly, the effect of transfection with WISP3 in cancer cell invasion was striking. Using the sea urchin invasion assay, a system that closely resembles the extracellular matrix that cells encounter *in vivo*, the WISP3 transfectants did not invade, when compared to controls. Furthermore, transfection with WISP3 cDNA greatly reduced tumor growth *in vivo*. Interestingly, transfection with WISP3 also resulted in a decrease in the rate of tumor uptake.

Another feature that makes IBC unique is its high angiogenic and angioinvasive potential. We proved that WISP3 suppressed pathologic neovascularization by reducing the levels of key pro-angiogenic factors, FGF2, VEGF, and IL-6.

Although the mechanism of action of the CCN proteins in general and of WISP3 in particular has not been elucidated, there are several candidates. The first of these could be by regulating nuclear cycling and/or apoptotic functions (Lopez-Bermejo *et al.*, 2000; Sprenger *et al.*, 1999). We observed a marked morphologic change in the SUM149 cell line after transfection with WISP3 cDNA. Histopathologic study of the tumors developed in nude mice revealed that those derived from the WISP3 transfectants had a significant reduction in the mitotic activity, as well as better differentiation and less cellular and nuclear pleomorphism. To better understand these morphologic changes, we studied the levels of key cell cycle regulators and apoptosis. The levels of the cell cycle inhibitors $p27^{kip1}$ and $p21^{waf1}$ were markedly increased by WISP3 expression, and cyclin E was concordantly decreased. Consistent with these cell-cycle specific gene expression changes, the percentage of apoptotic cells was also increased in the WISP3 transfectants. These data suggest that in the mammary gland, WISP3 expression may inhibit cancer cell growth through modulation of the cell-cycle regulation and apoptosis.

A second possible mode of tumor suppression activity for WISP3 is by regulating angiogenesis (Babic *et al.*, 1998; Perbal, 2001). As the tumor grows, it requires and promotes an increase in neovascularization. This activation of the angiogenic switch involves the up-regulation of angiogenic inducers and/or down-regulation of angiogenic inhibitors. Our results show that WISP3 expression induces a reduction in the level of key pro-angiogenic factors which results in a decrease in functional neovascularization.

A third mechanism of WISP3 tumor suppression could be by binding IGF-like ligands. WISP3 has the modular architecture of the CCN proteins and consists of four domains: an IGF-binding protein domain, a Von Willebrand factor domain, a thrombospondin 1 domain, and a C terminal domain (Babic *et al.*, 1998; Hashimoto *et al.*, 1998; Hurvitz *et al.*, 1999; Pennica *et al.*, 1998; Perbal, 2001). The N-terminal domain includes the first 12 cysteine residues and contains the highly conserved IGF binding consensus sequence (GCGCCXXC) which provides the proper folding of the protein to bind IGF (Byun *et al.*, 2001; Kim *et al.*, 1997; Oh *et al.*, 1996). Although this sequence is believed to be required for IGF-binding, the actual binding site may be slightly downstream of it (Imai *et al.*, 2000; Kalus *et al.*, 1998). The thrombospondin type 1 domain (TSP1) is presumably involved in inhibition

of angiogenesis (Perbal, 2001). The carboxy-terminal domain (CT) is present in most CCN proteins described to date and forms a cysteine knot. The protein is folded into two highly twisted antiparallel pairs of beta-strands and contains three disulfide bonds that may participate in dimerization and receptor binding. The CT domain does not appear to be as critical in IGF binding as the N-terminal region that contains the IGF-binding protein motif (Imai *et al.*, 2000; Kalus *et al.*, 1998). Although WISP3 may function in tumorigenesis through modulation of IGF binding, this is less likely since IGF-binding activity could not be demonstrated for other members of the CCN family (Perbal, 2001). We are currently investigating this possibility in mammary tissue.

In summary, we have proven that restoring expression of WISP3 into an aggressive IBC cell line, SUM149, inhibited cellular proliferation, drastically decreased angiogenesis, inhibited invasion, and decreased anchorage-independent growth, all hallmarks of malignant transformation. Restoration of WISP3 expression resulted in a morphologic alteration and induced tumor cell apoptosis with concordant molecular changes in cell cycle specific gene, $p27^{kip1}$ and $p21^{waf1}$. Finally, when SUM149 cells transfected with WISP3 were injected into female athymic nude mice, the rate of tumor formation and the tumor volume were markedly decreased when compared to controls. These studies demonstrate for the first time that WISP3 has strong tumor suppressor function in the mammary gland. Given the seemingly specific role that WISP3 plays in the development of IBC, our work may lead to the development of novel therapies that replace or mimic WISP3 function to treat this particularly challenging form of breast cancer.

Materials and methods

Cell culture

The derivation of SUM149 cell line has been described previously by Ethier *et al.* (1993). This cell line was developed from a human primary inflammatory breast cancer and has lost WISP3 expression (Figure 1, lane F). Cells were cultured in Ham's F-12 medium supplemented with 5% FBS, hydrocortisone (1 μg/ml), insulin (5 μg/ml), fungizone (2.5 μg/ml), Gentamycin (5 μg/ml) and each of Penicillin/streptomycin (100 μ/ml) at 37G under 10% CO_2.

Construction of expression vector and stable transfection

Total RNAs were isolated from human mammary epithelial cells (HME) using a Trizol kit (Life Technologies, Inc., Gaithersburg, MD, USA). First-strand cDNA synthesis was performed using 1 μg of total RNA with AMV reverse transcriptase (Promega, Madison, WI, USA) and oligo (dT) as a primer. Two μl of the reaction mixture were used for PCR amplification. Human WISP3 cDNA was amplified by PCR using the following forward and reverse primers: 5′-ATGCAGGGGCTCCTCTTCTGC-3′ and 5′-ACTTTTCCC-CCATTTGCTTG-3′, under the following conditions: denaturing for 1 min at 94°C, annealing for 1 min at 58°C, and elongation for 2 min at 72°C for 35 cycles. The PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). The 1.1 kb full-length cDNA encoding WISP3 was excised by *Eco*RI and subcloned into the *Eco*RI site of pFLAG-CV4 vector (Sigma, Saint Louis, MO, USA). The insert was confirmed by DNA sequencing. The plasmids were purified. Subsequently, the SUM149 cells were transfected with pFLAG-WISP3 and pFlag control vector by FuGene TM 6 transfection reagent (Roch-Boehringer Mannheim, Mannheim, Germany), and selected in the medium containing 150 μg/ml G418. The cells surviving during selection were expanded and maintained in the selected medium.

Western immunoblots

Western immunoblots were performed using the following primary antibodies: WISP3 polyclonal antibody (gift from Dr Matthew Warman), $p27^{kip1}$, $p21^{waf1}$, PCNA (Zymed, San Francisco, CA, USA) cyclin E (SC-247, Santa Cruz, CA, USA), cyclin D1 (SC-246, Santa Cruz, CA, USA), and actin (Sigma Chemical Co.). Cells were lysed in RIPA buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS. 0.1 mg/ml PMSF, 1 mM sodium orthovanadate, and 0.3 mg/ml aprotinin; Sigma Chemical Co.). Whole cell lysates (50 μg) were boiled in Laemelli buffer for 5 min, separated by 10% SDS–PAGE and transferred to PVDF membrane. Non-specific binding was blocked by incubation with 1.5% BSA in Tris-buffered saline with 0.5% Tween-20 (Sigma Chemical Co.). Antibody-antigen complexes were detected with an enhanced chemiluminescence kit (ECL, Amersham, Arlington Height, IL, USA) following the manufacturer's instructions.

Anchorage-independent growth

Cells from four SUM149/WISP3 stable clones and controls were seeded at a density of 1×10^3 per 35-mm plated in 0.3% agar and Ham's F-12 supplemented with 10% FBS, and plated on a base of 0.6% agar, Ham's F-12 and 10% FBS. Each assay was performed in triplicate. Plates were maintained at 37°C under 10% CO_2 for 3 weeks. Colonies greater than or equal to 100 μm in diameter were counted.

Annexin V-FITC binding assay

This assay was performed using the Apo Alert Annexin V kit (Clontech, CA, USA). Three clones of stably transfected SUM149/WISP3 and SUM149/Flag control cells (1×10^6) were trypsinized, washed and incubated in binding buffer (1 ×) containing 1 μg/ml of Annexin V-FITC conjugate (Clontech, CA, USA) for 10 min in the dark. Cells were next stained with propidium iodide (20 μg/ml) and analysed by flow cytometry (Martin *et al.*, 1995).

Monolayer growth rate

Monolayer culture growth rate was determined by qualitative measurement of the conversion of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Chemical Co.) to a water insoluble formazan by viable cells. Three thousand cells in 200 μl of culture medium were plated in 96-well plates and grown under normal conditions. Cultures were assayed at 0, 1, 3, 4 and 5 days by the addition of MTT and incubation for 1 h at 37°C. The MTT containing medium was aspirated and 100 μl DMSO (Sigma Chemical Co.) was added to lyse the cells and solubilize the formazan. Absorbance values of the lysates were determined on a Dynatech MR 5000 microplate reader at 540 nm.

Invasion and intrainvasion

To study invasion, the sea urchin extracellular membrane (SU-ECM) invasion assay was used (Livant *et al.*, 1995). Cells were layered onto sea urchin embryo extracellular basement membrane and allowed to invade for 4 h. The cells on the SU-ECM were fixed using paraformaldehyde and visualized under a microscope. Mean invasion percentages were calculated from two independent analyses of 50–100 randomly chosen cells in contact with the surfaces of the basement membranes.

Angiogenesis and angiogenic factor analysis

To study whether WISP3 modulated the angiogenic activity of IBC, the levels of key pro-angiogenic factors known to be secreted by IBC (VEGF, FGF2, IL-6, and IL-8) were measured in the cell supernatants by ELISA (van Golen *et al.*, 2000a). A functional analysis of angiogenesis of the conditioned media was performed. We used the rat aortic assay in which we assessed the amount of new blood vessel growth elicited by the conditioned medium of the WISP3 transfected cells (Nissanov *et al.*, 1995). Approximately 1-mm thick rings were cut from the aorta of a freshly sacrificed rat and embedded into Matrigel in the center of a 35-mm dish. Vessel outgrowth from the aorta into the Matrigel was visually quantified by counting the branching points in six randomly chosen fields for the SUM149/WISP3 and control SUM149/flag cells under a light microscope and compared at 7 and 10 days after planting. Statistical analysis was performed using the *t*-test.

In vivo *tumor formation*

Groups of 5–11, 10-week old, nude, athymic female mice were orthotopically injected into the mammary fat pad with 2×10^6 cells. Each group was injected with either a control (SUM149/Flag, or wild-type SUM149) clone, or one of two WISP3-expressing clones. The clones were selected according to their anchorage-independent growth characteristics. Mice were then monitored weekly for tumor formation for 8 weeks and for signs of wasting and chachexia as surrogates of survival for 18 weeks. If tumors were present, tumor volume was calculated using the formula $(l \times w^2)/2$ (where l = length and w = width of tumor). Rates of tumor formation were analysed by the Kruskal-Wallis method; and tumor volumes were assessed between the groups by the Mann–Whitney *U*-test. After the mice developed signs and symptoms of chachexia, they were sacrificed and their tumors were removed. A portion of each tumor (1/3) was digested with 0.1% collagenase (Type I) and 50 μg/ml DNase (Worthington Biochemical Corp. Freehold, NJ, USA), and RNA was collected for RT–PCR analysis. The remainder of the tumor tissues were used for histopathological study. After removal of the tumors, part of the tissues were fixed in 10% buffered formalin and processed for histopathological evaluation by paraffin-embedding and hematoxylin and eosin staining. Histological features studied included degree of anaplasia, mitotic activity, presence and amount of necrosis, and degree of differentiation (e.g. glandular formation).

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Characterization of RhoC Expression in Benign and Malignant Breast Disease

A Potential New Marker for Small Breast Carcinomas with Metastatic Ability

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The most important factor in predicting outcome in patients with early breast cancer is the stage of the disease. There is no robust marker capable of identifying invasive carcinomas that despite their small size have a high metastatic potential, and that would benefit from more aggressive treatment. RhoC-GTPase is a member of the Ras-superfamily and is involved in cell polarity and motility. We hypothesized that RhoC expression would be a good marker to identify breast cancer patients with high risk of developing metastases, and that it would be a prognostic marker useful in the clinic. We developed a specific anti-RhoC antibody and studied archival breast tissues that comprise a broad spectrum of breast disease. One hundred eighty-two specimens from 164 patients were used. Immunohistochemistry was performed on formalin-fixed tissues. Staining intensity was graded 0 to 3+ (0 to 1+ was considered negative and 2 to 3+ was considered positive). RhoC was not expressed in any of the normal, fibrocystic changes, atypical hyperplasia, or ductal carcinoma *in situ*, but was expressed in 36 of 118 invasive carcinomas and strongly correlated with tumor stage ($P = 0.01$). RhoC had high specificity (88%) in detecting invasive carcinomas with metastatic potential. Of the invasive carcinomas smaller than 1 cm, RhoC was highly specific in detecting tumors that developed metastases. RhoC expression was associated with negative progesterone receptor and HER-2/neu overexpression. We characterized RhoC expression in human breast tissues. RhoC is specifically expressed in invasive breast carcinomas capable of metastasizing, and it may be clinically useful in patients with tumors smaller than 1 cm to guide treatment. *(Am J Pathol 2002, 160:579–584)*

Breast cancer is the most common type of life-threatening cancer, and the second most common cause of cancer-related deaths of women in the Western world. The most important factor in predicting patient outcome is the stage of the disease.[1–3] Although in general, the more aggressive, the more rapidly growing, and the larger the primary neoplasm, the greater the likelihood that it will metastasize or already has metastasized, this is not always the case. There are many small breast cancers with a highly aggressive behavior and discouraging outcome that remain undertreated because there is no marker capable of identifying them.

RhoC-GTPase is a member of the Ras-superfamily of small guanosine triphosphatases (GTPases). Activation of Rho proteins leads to assembly of the actin-myosin contractile filaments into focal adhesion complexes that lead to cell polarity and facilitate motility.[4–6] Our laboratory has detected overexpression of RhoC mRNA in advanced breast cancers by *in situ* hybridization, and subsequently characterized RhoC as a transforming oncogene for human mammary epithelial cells, whose overexpression results in a highly motile and invasive phenotype that recapitulates the most lethal form of locally advanced breast cancer, inflammatory breast cancer.

We hypothesized that, given the known functions of the RhoC proteins, RhoC expression would be a good marker to identify breast cancer patients with highly aggressive and motile tumors and guide therapeutic interventions before the development of metastases. Immunohistochemistry is a reproducible and technically simple procedure that would allow testing for RhoC protein expression in the clinical setting. We set out to characterize the expression of RhoC protein in normal, benign, premalignant, and malignant breast disease, with special focus on small (<1 cm) invasive carcinomas with high metastatic potential and/or known metastases.

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Materials and Methods

Tissue Specimens

We evaluated 182 specimens from 164 patients. Breast tissues were obtained from surgical resections and biopsies from the breast and sites of distant metastases. These cases were selected from the surgical pathology files at the University of Michigan, reviewed by the study pathologist (CGK), and placed in the following pathological categories: normal breast parenchyma (5 cases), fibrocystic changes (5 cases), fibroadenomas (3 cases), atypical ductal hyperplasia (7 cases), ductal carcinoma *in situ* (11 cases), invasive ductal carcinoma (114 cases), other types of invasive carcinoma (lobular, 13 cases; mucinous, 6 cases; medullary, 2 cases). In addition, 16 metastatic deposits were analyzed, 9 of which had their corresponding primary tumor to compare. Invasive carcinomas were subdivided by stage into stages I, II, III, and IV. Hormonal receptor status and immunohistochemical staining for HER2/neu was available for most patients. Clinical follow-up information was available for all patients. Patient identifiers were removed for subsequent analyses.

Development of RhoC-Specific Antibody

Because RhoC-GTPase has high homology to other members of the Rho family, RhoA and RhoB, both at the cDNA and the protein level, most available antibodies are cross-reactive with RhoA, RhoB, and RhoC. To attempt to develop an antibody specific for RhoC and not for other Rho family members, a peptide representing a unique epitope was synthesized at the University of Michigan Protein Core. The C-terminal region peptide (GLVQVRKNKRRRGCPIL) was chosen because of its uniqueness and antigenic potential. After injection in rabbits, immune sera were obtained following standard techniques. Western blot confirmed the specificity of the antibody for RhoC (Figure 1). Specifically no cross-reaction was observed to recombinant RhoA. To further prove the high specificity of the antibody for RhoC protein, we performed a competition assay by incubating the anti-RhoC antibody with increasing concentrations of the RhoC peptide in 6 ml of 0.3% bovine serum albumin for 6 hours at 4°C. Subsequently a Western blot was performed following standard procedures (Figure 2A). As illustrated in Figure 2B, the specificity of the antibody was also checked by blocking the binding by incubating overnight with a 10-fold molar excess of the RhoC peptide.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections that were cut 4-μm thick and stained with polyclonal anti-RhoC antibody. The antibody was titered and used at a 1:1500 dilution for 30 minutes at room temperature, with no previous antigen retrieval. The detection reaction followed the DAKO En-

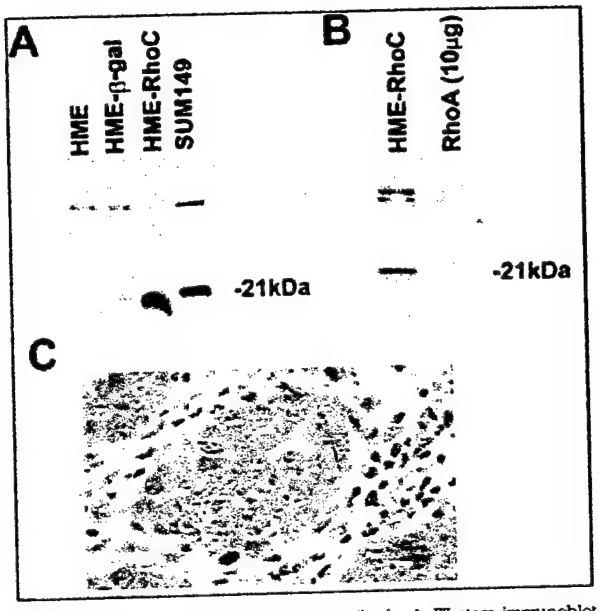
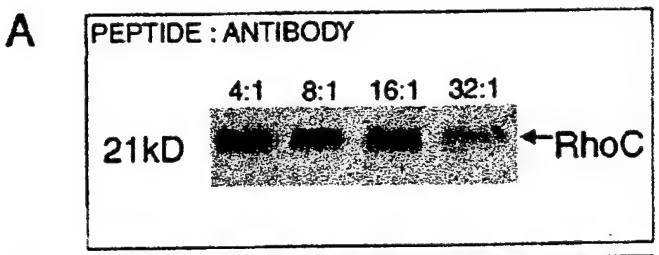


Figure 1. Development of specific RhoC antibody. **A:** Western immunoblot showing the specificity of the RhoC antibody for RhoC, without cross-reaction with recombinant RhoA, shown in **B**. **C:** Immunohistochemistry using RhoC antibody in a tumor xenograft developed by injecting human mammary epithelial cells overexpressing RhoC in the mammary fat pad of female athymic nude mice. Strong staining of the cytoplasm of the neoplastic cells. HME, human mammary epithelial cells; HME-β-gal, transfection controls; HME-RhoC, HME cells transfected with RhoC gene; SUM149, cell line derived from a patients with inflammatory breast cancer that overexpresses RhoC.

vision+ System peroxidase kit protocol (DAKO, Carpinteria, CA). Diaminobenzidine was used as chromogen and hematoxylin was used as counter stain. As positive controls we used tumor xenografts from a cell line known to overexpress RhoC (SUM 149) and from human mammary epithelial cells transfected with RhoC, and patient tumor specimens previously demonstrated to overexpress RhoC by *in situ* hybridization.[7] Negative controls were done by omitting the primary antibody.

Interpretation of Stains

Because RhoC protein interacts with the contractile cytoskeleton of the cell and is localized to the submembrane space, cytoplasmic stain was expected. Not surprisingly, myoepithelial cells and vascular smooth muscle cells were strongly positive in all cases, serving as consistent internal positive controls (Figure 3). The intensity of cytoplasmic staining was scored as 0 to 3+, by comparison to the positive internal controls. This scoring system has been previously validated.[7,8] Diffuse, moderate to strong cytoplasmic staining characterized RhoC-positive cells (scores 2+ and 3+) (Figure 3; C, D, and E). RhoC-negative cells were devoid of any cytoplasmic staining or contained faint, equivocal staining (scores 0 and 1+) (Figure 3. B and F).



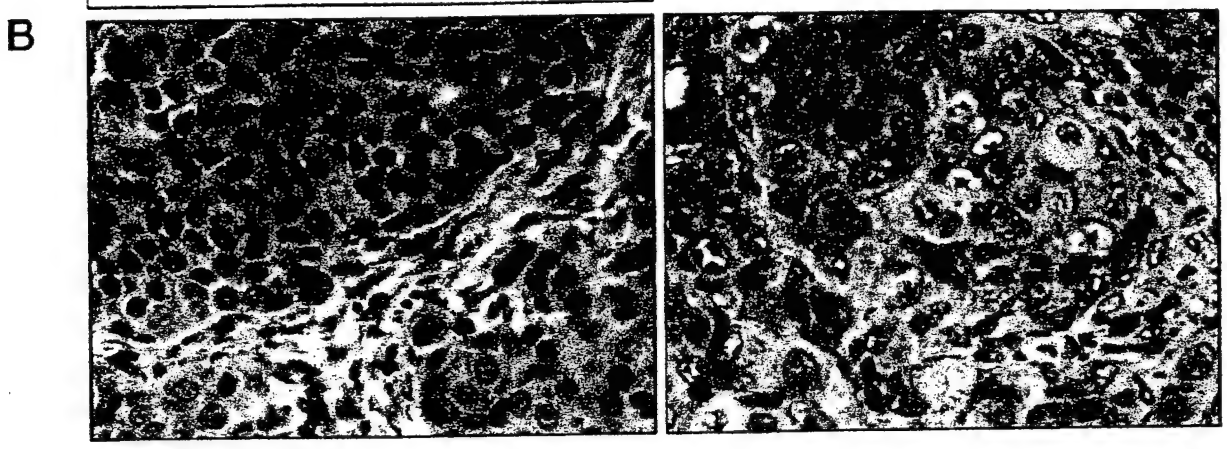


Figure 2. Demonstration of the specificity of the anti-RhoC antibody. **A:** Western immunoblot with increasing peptide:antibody concentrations. **B:** Competition assay by immunohistochemistry before (**left**) and after (**right**) addition of a 10-fold molar excess of RhoC peptide (original magnification, ×400).

Statistical Analysis

The chi-square test was used to assess differences in RhoC expression between invasive carcinoma of different stages. Fisher's exact test was used to study the relationship between RhoC expression and development of metastases, to study whether RhoC expression was significantly different in inflammatory breast cancer (IBC) primary tumors *versus* lymphatic emboli, and to determine the association between RhoC expression and estrogen receptor, progesterone receptor, and HER2/neu status.

Results

RhoC Is Not Expressed in Normal Breast, Fibrocystic Changes, or Preinvasive Breast Disease

We studied five cases of normal breast parenchyma and five cases of fibrocystic changes obtained from reduction mammoplasties and breast biopsies, respectively. RhoC was not detectably expressed in the ductal epithelium in any cases (Figure 3A). In addition, the three fibroadenomas tested revealed no RhoC protein expression. No RhoC protein expression was seen in any cases of atypical ductal hyperplasia (seven cases) low-grade ductal carcinoma *in situ* (6 cases), or high-grade ductal carcinoma *in situ* (five cases) (Figure 3B). All cases however had consistent and strong RhoC staining of myoepithelial cells and vascular smooth muscle, which served as internal positive control (Figure 3).

RhoC Is Expressed in Invasive Carcinomas with Metastases and its Expression Increases with Primary Tumor Stage

Moderate and strong RhoC protein expression (scores 2+ and 3+) was detected in 36 of the 114 (32%) primary invasive ductal carcinomas. When invasive ductal carcinomas were categorized by stage, a strong correlation was found between RhoC protein expression and tumor stage (chi-square test, $P = 0.01$) (Figure 4). The relationship of RhoC expression to the development of metastases is illustrated in Figure 5. Of the 36 invasive ductal carcinomas that expressed RhoC, 30 (83%) had axillary lymph node or distant metastases, and 6 (17%) did not metastasize. The specificity and sensitivity of RhoC in predicting the development of metastases was 88% and 47%, respectively. The positive and negative predictive values were 83% and 56%, respectively.

Eighty percent of primary IBCs expressed RhoC protein. The intralymphatic tumor emboli seen in the skin biopsies obtained from IBC patients however were negative (Fisher's exact test, $P < 0.001$) (Figure 2F). Regardless of the pathological tumor stage, RhoC expression was not detected in primary invasive lobular carcinoma, invasive typical medullary carcinomas, or invasive mucinous (colloid) carcinomas.

RhoC Expression in Small Invasive Carcinomas with High Metastatic Potential

When invasive ductal carcinomas were separated by primary tumor size, 20 tumors (18%) were smaller than 1

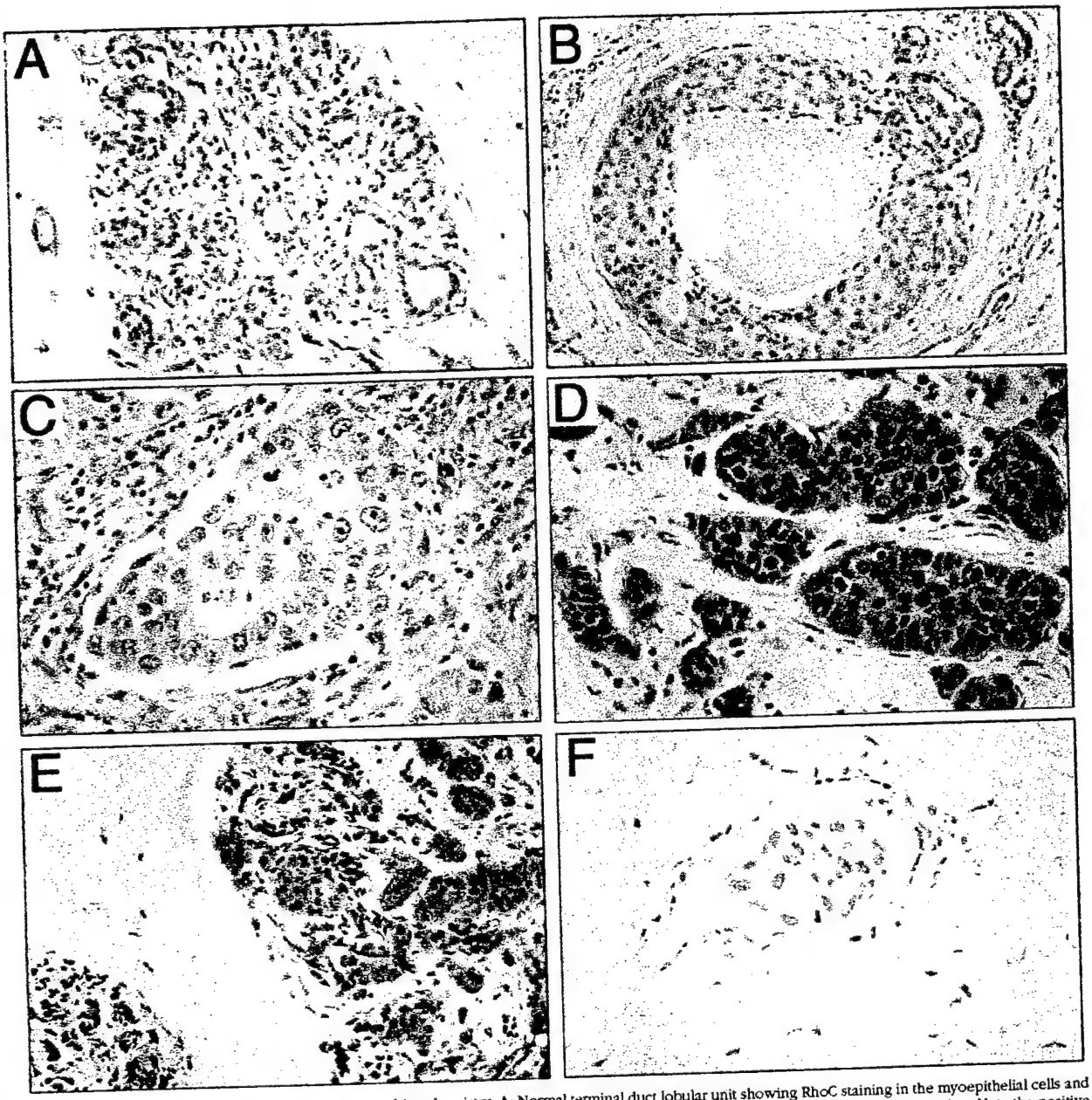


Figure 3. Results of RhoC protein detection by immunohistochemistry. **A:** Normal terminal duct lobular unit showing RhoC staining in the myoepithelial cells and vessels in contrast to the negative normal epithelial cells. **B:** High-grade ductal carcinoma *in situ* with central necrosis and no RhoC expression. Note the positive staining of the surrounding blood vessels and occasional myoepithelial cell that serve as positive internal controls. **C:** Primary invasive ductal carcinoma that measures 0.6 cm with RhoC protein expression (2+). This carcinoma developed axillary lymph node metastases. **D:** Primary stage 3 invasive ductal carcinoma with strong (3+) cytoplasmic staining for RhoC protein. **E:** Metastatic ductal carcinoma from the breast to the iliac bone showing strong RhoC expression. **F:** Intralymphatic carcinomatous embolus in a dermal lymphatic vessel in a patient with the clinical and pathological features of inflammatory breast cancer with no RhoC protein expression. Note the positive staining of the endothelial cells.

cm (Figure 6). Of these, 13 had no metastases, 6 metastasized to axillary lymph nodes, and 1 developed metastases to axillary lymph nodes and colon. RhoC was moderately (2+) expressed in 3 of 7 (43%) tumors that metastasized and not expressed in 12 of 13 (92%) invasive carcinomas that did not metastasize (Fisher's exact test $P = 0.10$) (Figure 6). RhoC had a specificity of 92% and a sensitivity of 43% in detecting tumors that have metastatic ability. RhoC's positive and negative predictive values were 75%.

RhoC Protein Expression in Breast Cancer Metastases

Of the 14 distant metastases [liver ($n = 3$), cerebellum ($n = 1$), bone ($n = 4$), bone marrow ($n = 1$), lung ($n = 2$), large intestine ($n = 2$), ovary ($n = 1$), and uterus ($n = 1$)], 7 (50%) expressed RhoC protein (Figure 2E). After these cases were categorized by histological type, RhoC was expressed in five of eight (62.5%) metastases from inva-

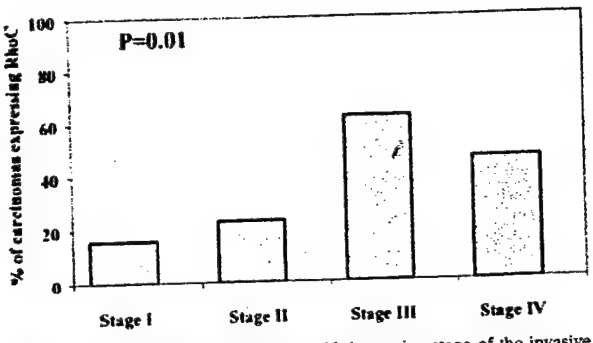


Figure 4. Increasing RhoC expression with increasing stage of the invasive breast carcinoma.

sive ductal carcinomas, in two of five (40%) metastases from invasive lobular carcinomas, and it was negative in the one metastasis from a medullary carcinoma.

RhoC Expression Is Associated with Negative Progesterone Receptor and Overexpression of HER2/neu

Table 1 summarizes the results. We observed that tumors that expressed RhoC were more frequently negative for progesterone receptor, and overexpressed Her-2/neu by immunohistochemistry. Although the associations did not reach statistical significance they suggest that RhoC expression seems to be associated with well-known predictors of patient outcome. No association was found between RhoC and estrogen receptor expression.

Discussion

The Rho (Ras homology) gene was first isolated from aplysia and has been shown to be highly conserved throughout evolution. RhoC-GTPase is involved in cytoskeletal reorganization, specifically in the formation of actin stress fibers and focal adhesion contacts.[4–6] Our laboratory has recently demonstrated that RhoC is overexpressed in IBC tumors by *in situ* hybridization,[9] and has

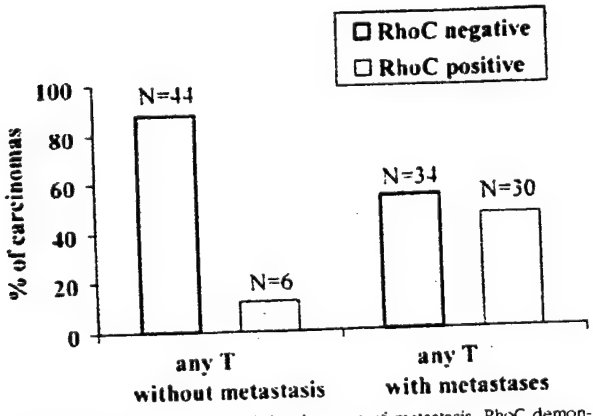


Figure 5. RhoC expression and development of metastasis. RhoC demonstrated a specificity of 88% and a sensitivity of 47% in detecting invasive carcinomas that metastasized.

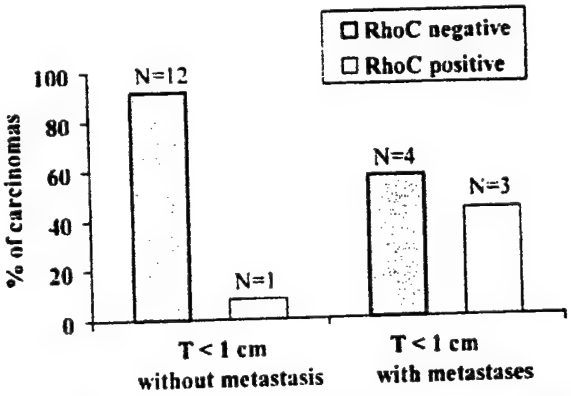


Figure 6. RhoC expression identifies a group of invasive ductal carcinomas smaller than 1 cm that developed axillary lymph node and distant metastases. RhoC demonstrated a specificity of 92% and a sensitivity of 43% in detecting invasive carcinomas smaller than 1 cm that metastasized.

shown that overexpression of RhoC induced the malignant transformation of immortalized human mammary epithelial cells by producing an aggressive, highly motile, and invasive phenotype that partially recapitulates the behavior of IBC in humans.[10]

Based on these results, we hypothesized that expression of RhoC would identify invasive carcinomas that despite their small size have a highly invasive and metastatic potential, and thus develop into a useful screening tool to be used in the clinical arena. We also hypothesized that RhoC may be a new prognostic marker in patients with breast cancer. To test our hypotheses, we developed a specific and sensitive polyclonal antibody directed against the RhoC protein that can be used for immunohistochemistry, and set out to characterize RhoC protein expression in a wide spectrum of breast pathology, from normal, benign lesions, premalignant and *in situ* carcinomas, to invasive carcinomas of the breast.

From our results several important conclusions can be drawn. First, RhoC expression may not be an early event in the development of non-IBC breast cancer, but a later genetic alteration that occurs once the cancer cells have acquired invasive capabilities. We showed that RhoC is exclusively expressed in invasive carcinomas and not in normal breast, atypical intraductal hyperplasia, or ductal carcinoma *in situ*. In IBC, the most lethal type of locally advanced breast cancer that is highly metastatic from its inception, RhoC seems to occur early in its development because 80% of all primary IBCs expressed the protein. These results support our previous observations that RhoC is consistently overexpressed in IBC.[9] Interestingly, none of the dermal lymphatic tumor emboli expressed RhoC. A possible explanation may be that endolymphatic tumor emboli are cohesive clumps of cancer cells and do not need to acquire motile capabilities until they reach the site of metastases, at which point the tumor cells extravasate and invade new tissues. This argument is supported by a previous study from our laboratory that showed that intralymphatic tumor emboli strongly express E-cadherin, an epithelial cell-cell adhesion molecule that enables the cancer cells to form tightly cohesive tumor emboli.[11]

Table 1. Relationship between RhoC Protein Detection by Immunohistochemistry, Estrogen and Progesterone Receptor Status, and HER-2/neu Overexpression

	RhoC protein expression		
	Negative (0–1–)	Positive (2–3+)	*P**
Estrogen receptor, *n* (%)			
Negative	21 (64%)	12 (36%)	0.49
Positive	51 (72%)	20 (28%)	
Progesterone receptor, *n* (%)			
Negative	29 (62%)	18 (38%)	0.14
Positive	43 (75%)	14 (25%)	
HER-2/neu, *n* (%)			
Not overexpressed	39 (83%)	8 (17%)	0.08
Overexpressed	28 (67%)	14 (33%)	

*P, Fisher's exact test.

Second, RhoC seems to be a marker of metastatic potential in breast cancer. We demonstrated that nearly half of the invasive ductal carcinomas that developed metastases expressed RhoC (30 of 64 cases, 47%), in contrast to very few of the invasive carcinomas without metastases (6 of 50 cases, 12%). RhoC demonstrated a high specificity (88%) in detecting which tumors have the ability to metastasize. These results are in concordance with previous data showing that overexpression of RhoC-GTPase in immortalized human mammary epithelial cells leads to a motile and invasive phenotype able to develop highly metastatic tumors when injected in nude mice.[10] Not surprisingly, we found that RhoC-GTPase expression increases with the stage of the invasive carcinoma.

Third, our results suggest that RhoC protein detection by IBC may be a useful tool capable of identifying small invasive ductal carcinomas with high propensity to metastasize. Although the number of cases in our study is small, RhoC was highly specific (specificity of 92%) in detecting small invasive carcinomas with metastatic potential. Forty-three percent of invasive carcinomas smaller than 1 cm that developed metastases expressed RhoC protein, in contrast to 8% of the small tumors that did not metastasize. We are currently expanding the number of cases to further define these observations. This potential use of RhoC in the clinical setting may have a profound impact in the management of breast cancer patients. Specifically, detection of RhoC expression may identify patients who will benefit from an axillary lymph node dissection given the high risk of metastases. RhoC expression may also suggest the use of chemotherapy in patients with small invasive carcinomas at high risk of metastases.

Our data revealed an association between RhoC expression, Her-2/neu overexpression, and loss of progesterone receptor in invasive ductal carcinomas, both well-established predictors of patient outcome. Although primary invasive lobular carcinomas did not express RhoC, 40% of their distant metastases expressed the protein, suggesting that RhoC expression may be involved in the metastatic process of this type of invasive carcinoma, but may be a later event than in non-IBC invasive ductal carcinomas. RhoC expression does not seem to play a role in the late stages of other uncommon forms of invasive breast cancer, including medullary and colloid (mucinous) carcinomas.

Because RhoC protein is associated with the contractile cytoskeleton of the cell, it is not surprising that it is detected by immunohistochemistry in myoepithelial cells and in the vascular smooth muscle cells. These two cell types served as consistent and strong (3+) internal positive controls for the antibody and were detected in all cases.

This study is the first examining the expression of RhoC-GTPase protein in a wide spectrum of normal breast and of breast disease. It is clear from the results that RhoC is a specific marker of metastatic disease in patients with breast cancer. Importantly, although our data are preliminary, it appears to identify a subset of patients with small primary tumors and high metastatic potential that would benefit from axillary lymph node staging and/or chemotherapy and that would remain otherwise unrecognized.

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Reversion of RhoC GTPase-Induced Inflammatory Breast Cancer Phenotype by Treatment with a Farnesyl Transferase Inhibitor[1]

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Abstract

Inflammatory breast carcinoma (IBC) is a highly aggressive form of locally advanced breast cancer that has the ability to invade and block the dermal lymphatics of the skin overlying the breast. In a previous series of studies, our laboratory identified overexpression of RhoC GTPase in >90% of IBCs and defined *RhoC* as a mammary oncogene involved in conferring the metastatic phenotype. RhoC GTPase is involved in cytoskeletal reorganization during cellular motility. Farnesyl transferase inhibitors (FTIs) were previously shown to be effective in modulating tumor growth in Ras-transformed tumor cells. Recently, studies have focused on RhoB as a putative non-Ras target of FTI action. In the present study, we assessed the effect of the FTI L-744,832 on RhoC-overexpressing IBC and RhoC-transfected human mammary epithelial (HME-RhoC) cells. Treatment of the SUM149 IBC cell line and HME-RhoC transfectants with the FTI L-744,832 led to reversion of the RhoC-induced phenotype, manifested by a significant decrease in anchorage-independent growth, motility, and invasion. Although RhoC expression and activation were not affected, RhoB levels were increased by FTI treatment. Transient transfection of geranylgeranylated RhoB (RhoB-GG) into the same cells reproduced the effects of the FTI, thus suggesting that FTI-induced reversion of the RhoC phenotype may be mediated by an increase in RhoB-GG levels. These data provide direct evidence that FTIs may find use in the clinic when directed against RhoC-overexpressing tumors and suggest appropriate biological markers to evaluate during FTI treatment.

Introduction

The term IBC[3] was first coined in 1924 by Drs. Lee and Tannenbaum to describe a phenotypically distinct form of locally advanced breast cancer (LABC) (1, 2). IBC is a fast-growing, highly invasive, and metastatic form of LABC, which is clinically characterized by primary skin changes (1–4). These primary skin changes are the result of blockage of the dermal lymphatics of the skin overlying the breast resulting in edema, *peau d' aurange,* and nipple retraction (1–4). At the time of diagnosis, nearly all tumors have spread to the regional lymph nodes and on close inspection, more than one-third of patients have gross distant metastases (1–4). Despite aggressive multimodality treatments, the 5-year disease-free survival rate for women with IBC is <45%, making IBC the deadliest form of breast cancer (1–4).

During investigation of the genetic mechanisms responsible for the unique IBC phenotype, our laboratory identified overexpression of RhoC GTPase in >90% of IBCs (5). RhoC GTPase is a member of the Ras-homology family of small GTP-binding proteins and is responsible for cytoskeletal reorganization during cellular motility (6–10). RhoC belongs to a highly homologous subfamily comprised of RhoA, RhoB, and RhoC (11). Although these family members have >90% sequence homology to one another, their roles in the cell are distinct (11). To determine the contribution of RhoC GTPase overexpression to the IBC phenotype, we generated stable RhoC-overexpressing HME cell lines (HME-RhoC) (12). The HME-RhoC clones nearly recapitulated the invasive features of the IBC phenotype. Specifically, the cells grew under anchorage-independent conditions and produced tumors when orthotopically injected into athymic nude mice (12–14). The cells were highly motile and invasive and produced conditioned medium rich in pro-angiogenic cytokines *in vitro* (12–14). Taken together, these data demonstrate that overexpressed, active *RhoC GTPase* is a mammary oncogene leading to advanced disease.

Regulation of the GTPase activity of both the Ras and the Rho proteins is achieved through interactions of GAPs, GDIs, GDF, and GEFs (15, 16). RhoA, RhoC, and a fraction of RhoB are geranylgeranylated, and the remaining portion of RhoB is farnesylated (17–19). For the Rho proteins to enter the GDP/GTP cycle they must be transported and localized to the membrane (19, 20). GTP binding produces a conformational

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[3] The abbreviations used are: IBC, inflammatory breast cancer; FTI, farnesyl transferase inhibitor; HME, human mammary epithelial (cells); FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; PI, propidium iodide; RhoB-GG, geranylgeranylated RhoB; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; PI3K, phosphatidylinositol 3′-kinase; β-gal, β-galactosidase; GAP, GTPase-activating proteins; GDI, GDP-dissociation inhibitor; GDF, GDI dissociation factor; GEF, guanine nucleotide exchange factor.

change in the GTPase, thereby allowing interaction with downstream effector proteins (15, 21). Hydrolysis of GTP to GDP by the intrinsic Rho GTPase activity modulates the interaction with the effector protein (22, 23). The GTPase activity is greatly increased by activated GAPs, thus leading to increased hydrolysis of GTP (24). The entire process is balanced by the GDIs, which prevent GDP dissociation by binding to the prenylation group of the GTPase and sequestering the complex in the cytoplasm (25). The GTPase is subsequently liberated from the GDI by GDFs, closing the cycle (25).

In light of evidence demonstrating that FTIs can target non-Ras molecules, such as the RhoB protein, and recent work suggesting that RhoB alterations, specifically, the accumulation of RhoB-GG, may interfere with transforming Rho signals, we sought to test the effect of FTIs on RhoC-transformed breast cells.

Materials and Methods

Cell Culture. Cell lines were maintained under defined culture conditions for optimal growth in each case as described previously (26–28). E6/E7 immortalized HME cells (29) were grown in 5% FBS (Sigma Chemical Co., St. Louis, MO)-supplemented Ham's F-12 medium (JRH BioSciences, Lenexa, KS) containing insulin, hydrocortisone, epidermal growth factor, and cholera toxin (Sigma Chemical Co.). Stable HME transfectants containing either the human *RhoC GTPase* or control *β-gal* genes were maintained in the described medium supplemented with 100 μg/ml hygromycin (LifeScience Technologies; Gaithersburg, MD) as described previously (12, 13). The SUM149 IBC cell line was grown in 5% FBS-supplemented Ham's F-12 medium containing insulin and hydrocortisone. The HME cells were characterized as being keratin 19 positive, thus ensuring that they are from the same differentiation lineage as the SUM149 IBC tumor cell line. For FTI treatment, actively growing cells were treated with 25 μM FTI L-744,832 and harvested 48 h later. Cell viability was assessed prior to assays using a trypan blue exclusion assay. Harvested cells were washed in 10 ml of HBSS (LifeScience Technologies). A 100-μl aliquot was taken, diluted 1:1 with prediluted trypan blue (Sigma Chemical Co.), and counted on a hemacytometer.

Transient transfections were performed by growing cells in 100-mm plates until reaching 50% confluence. Expression constructs for wild-type RhoB, RhoB-GG, and a geranylgeranyl-deficient RhoB mutant were generated as described previously (30–32). The RhoB containing vectors or a vector control were introduced into the cells using FuGene6 transfection reagent (Roche, Indianapolis, IN) as described previously (12). Transient transfectants were used in biological assays 24 h after transfection.

Anchorage-independent Growth and Focus Formation. For anchorage-independent growth assays, a 2% stock of sterile low-melt agarose was diluted 1:1 with 2× MEM. Further dilution to 0.6% agarose was made using 10% FBS-supplemented Ham's F-12 medium complete with growth factors, and 1 ml was added to each well of a six-well plate as a base-layer. The cell layer was then prepared by diluting agarose to 0.3% and 0.6% with 10^3 cells (either untreated or 25 μM FTI L-744,832 for 24 h) in 2.5% FBS-supplemented Ham's F-12/1.5 ml/well. A 1-ml layer of medium was maintained on top of the agar to provide nutrients and, in the case of the treated cells, additional inhibitor. Colonies ≥100 μm in diameter were counted after a 2-week incubation at 37°C in a 10% CO_2 incubator.

A modified focus formation assay was performed by harvesting treated and untreated cells and plating at dilutions of 1000, 500, and 100 cells/35-mm dish. The cells were then cultured for 2 weeks at 37°C in a 10% CO_2 incubator. The plates were washed with 10 ml of PBS, fixed for 10 min with ice-cold methanol, and stained for 10 min with 2% methylene blue in 50% ethanol, and visible foci were counted.

Western Blot and RhoC Activation Analysis. Proteins were harvested from cell cultures using radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 0.3 mg/ml aprotinin; Sigma Chemical Co.) Ten-μg aliquots were mixed with Laemmli buffer, heat-denatured for 3 min, separated by SDS-PAGE, and transferred to nitrocellulose. Nonspecific binding was blocked by overnight incubation with 2% powdered milk in Tris-buffered saline with 0.05% Tween 20 (Sigma Chemical Co.). Immobilized proteins were probed using antibodies specific for RhoC GTPase (33), or RhoB GTPase (Cytoskeleton Inc. Denver, CO). Protein bands were visualized by ECL (Amersham-Pharmacia Biotech, Piscataway, NJ).

A RhoC activation assay was performed as described previously (34, 35). Cells grown to 40% confluence were incubated in the presence or absence of 25 μM FTI L-744,832 for 24 h. Proteins were harvested using GST-FISH buffer (34) and were centrifuged. The supernatant was mixed with a slurry of GST-rhotekin fusion protein bound to glutathione-Sepharose beads. Only GTP-bound Rho binds to the GST-rhotekin fusion protein. The mixture was centrifuged, separated by SDS-PAGE, transferred to nitrocellulose and probed using a RhoC-specific antibody (33). Protein bands were visualized by ECL and exposed to Hyperfilm (Amersham).

Semiquantitative RT-PCR. Total RNA was harvested from actively growing cells at 50% confluence using Trizol Reagent (Life Technologies), and cDNA was made using the AMV-reverse transcriptase kit (Promega, Madison, WI). RhoC and RhoB transcripts were PCR amplified from aliquots of cDNA using a 1:100 dilution of Rho-specific primers mixed with GAPDH primers. PCR products were then separated on a 1.2% TAE-agarose gel and were visualized by ethidium bromide. The relative intensity of the Rho and GAPDH bands was measured using an Alpha Imager 2200 (Alpha Innotech Co., San Leandro, CA).

Motility and Invasion Assays. Random motility was determined using a gold-colloid assay (36). Gold colloid was layered onto glass coverslips and placed into 6-well plates. Cells were seeded onto the coverslips and allowed to adhere for 1 h at 37°C in a CO_2 incubator (12,500 cells/3 ml in serum-free medium). To stimulate the cells, the serum-free medium was replaced with 5% FBS containing Ham's F-12 supplemented with growth factors and allowed to incubate for 3 h at 37°C. The medium was aspirated and the cells fixed using 2% gluteraldehyde (Sigma Chemical Co.). The cover-

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slips were then mounted onto glass microscope slides and areas of clearing in the gold colloid corresponding to phagokinetic cell tracks were counted.

The invasion assay was performed as described previously with minor modification (37). A 10-μl aliquot of 10 mg/ml Matrigel (BD Biosciences, Bedford, MA) was spread onto a 6.5-mm Transwell filter with 8 μm pores (Costar, Corning, NY) air-dried in a laminar flow hood, and reconstituted with a few drops of serum-free medium. The lower chamber of the Transwell was filled with either serum-free or serum-containing media. Cells were harvested and resuspended in serum-free medium with 0.1% BSA at a concentration of 3.75×10^5 cells/ml, and 0.5 ml was added to the top chamber. The chambers were incubated for 24 h at 37°C in a 10% CO_2 incubator. The cell suspension was aspirated, and excess Matrigel was removed from the filter using a cotton swab. The filters were then cut away from the Transwell assembly and fixed, gel side down, with methanol to a glass microscope slide, stained with H&E, and 20 random ×40 magnification fields were counted. The number of cells that had invaded into the serum-free medium-containing lower chambers were considered background and were subtracted from the number of invaded cells in the serum-containing samples.

Statistical analysis was performed using a two-tailed Student *t* test.

Apoptosis Assay. Cytofluorometric analysis of cell cycle distribution and apoptosis was performed by PI staining of nuclei as reported previously (38, 39). Briefly, cells were treated with 25 μM FTI L-744,832 (Merck) alone, 10 μM LY294002 (Calbiochem, San Diego, CA) alone, or a combination of both FTI L-744,832 and LY294002. Untreated and treated cells, 1×10^6, were harvested from 35-mm wells, washed once with ice-cold PBS (Fisher Scientific, Pittsburgh, PA) and pelleted; supernatants were removed and 500 μl of PI-hypotonic lysis buffer [0.1% sodium citrate, 0.1% Triton X, 100 μg/ml RNAse type I-A, 50 μg/ml PI (SIGMA)] were added. Samples were analyzed by flow cytometry after a 20-min incubation at 25°C.

Rhodamine-Phallodin Staining of Actin Filaments. Visualization of actin filaments was accomplished by staining the cells with a rhodamine-conjugated phallotoxin. Briefly, cells were grown on glass coverslips for 48 h and washed with PBS followed by fixation with 1:1 ice-cold acetone and methanol. After a 30-min incubation in PBS containing 1% BSA, 5 μl of methanolic rhodamine-phalloidin stock (Molecular Probes, Eugene, OR) were added to each coverslip and allowed to stain for 20 min at room temperature. After repeated washing with PBS, the coverslips were mounted onto glass microscope slides using Gel/Mount (Biomedia Co., Foster City, CA). Cells were visualized under a Zeiss scanning laser confocal microscope equipped with a 573-nm fluorescence filter.

Scanning Electron Microscopy. Cells (12,000) were fixed with buffered 2.5% glutaraldehyde for 1 h, rinsed, and post-fixed for an additional hour with buffered osmium tetroxide. After dehydration in ascending strengths of ethanol, the cells were critical-point dried, mounted onto standard SEM stubs, and gold-sputter coated. They were viewed using an AMRAY 1000-B Scanning Electron Microscope. AQ: J AQ: K

Results

Effect of FTI Treatment on RhoC-overexpressing Breast Cells. The ability of cells to grow in soft agar is a hallmark of malignant transformation (40). Previously, we found that RhoC overexpression led to the growth of mammary epithelial cells under anchorage-independent conditions (12, 14). As demonstrated in Fig. 1*A*, treatment of RhoC-overexpressing HME cells and the SUM149 IBC cell line with 25 μM FTI L-744,832 resulted in a significant decrease in anchorage-independent growth. Although the HME-β-gal control-transfected cells did not readily grow under anchorage-independent conditions, they were slightly affected by FTI treatment. The 80% decrease in anchorage-independent growth of the Rho-expressing cells did not correlate with a decrease in monolayer growth rate as determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown). It has been suggested that FTI treatment sensitizes cells to apoptotic death on treatment with the PI3K inhibitor LY294002 (31). However, in our system, we did not observe an increase in apoptosis in cells treated with FTI L-744,832 alone, LY294002 alone, or a combination of the two, as has been observed for Ras-transformed cells (data not shown). F1

To evaluate the effect of FTI treatment on RhoC-mediated cellular motility, we assessed the same treated cell lines in colloidal-gold random motility assay. Cells were seeded onto glass coverslips overlayed with a gold colloid and stimulated with serum to induce motility. Discernable and quantifiable tracks were left as the cells moved and phagocytized the gold colloid. At 24 h after stimulation, the SUM149 and HME-RhoC cells treated with FTI L-744,832 were 1.8- to 2-fold less motile than their untreated counterparts (Fig. 1*B*). Both the HME-β-gal control and the MCF10AT c1 (an MCF10A clone transfected with a constituitively active Ras; Ref. 41) were unaffected by FTI treatment. As determined by a trypan blue dye exclusion assay, the reduction in cell motility was not caused by a decrease in the number of viable cells (data not shown). AQ: L

As shown in Fig. 1C, when the FTI-treated cells were tested for their ability to invade through a Matrigel-coated filter in response to a chemoattractant, it was found that the SUM149 and HME-RhoC cells were 2-fold less invasive than the untreated cells. Again, the control cell lines, HME-β-gal and MCF10AT c1, were unaffected by FTI treatment.

Taken together, these data suggest that treatment of RhoC-overexpressing cells with a FTI leads to the inhibition of RhoC-mediated anchorage-independent growth, motility, and invasion without significantly affecting cell growth or viability.

As demonstrated in Fig. 2, *C* and *E*, rhodamine-phallodin staining for actin filaments shows a highly organized and polarized cytoskeleton in the RhoC-overexpressing cells. These actin bundles are lost or diminished on treatment with FTI L-744,832 (Fig. 2, *D* and *F*). Numerous focal adhesions were visible on the periphery of the treated cells. However, loss of cytoskeletal polarity led to morphological changes F2

F3

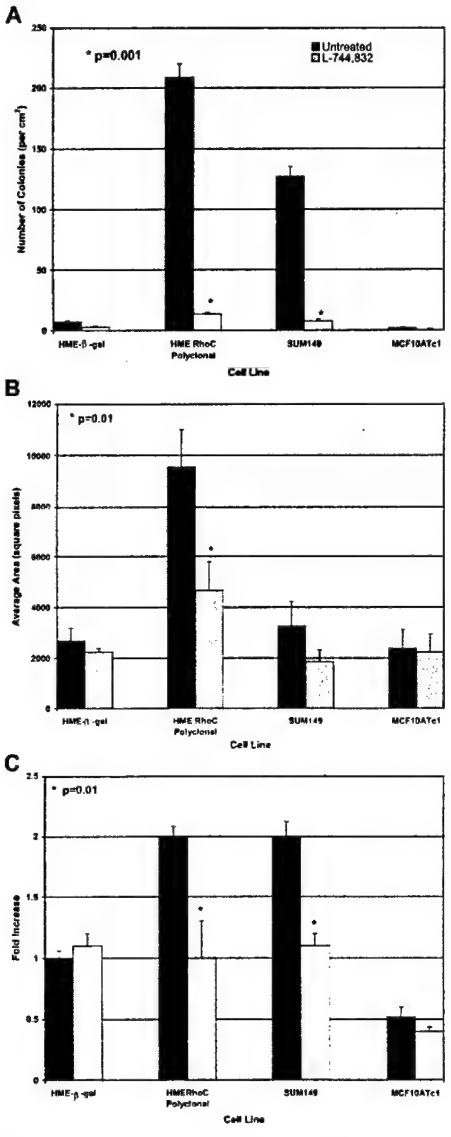

Fig. 1. A, comparison of anchorage-independent growth of untreated and FTI L-744,832 treated cell lines 0.6% soft agar. The growth of RhoC overexpressing cell lines, HME-RhoC and SUM149, were significantly inhibited (P = 0.001) by FTI treatment, which suggests a reversion of malignant transformation. *B* demonstrates a significant reduction in the motility of FTI-treated HME-RhoC cells. A reduction in motility, although not significant, was also observed for the SUM149 cells. Similarly, a significant reduction (P = 0.01) in the ability to invade through a Matrigel-coated filter was also observed for the HME-RhoC and SUM149 cells (*C*). None of the effects that resulted from treatment with FTI could be attributed to a decrease in viable cells, as determined by trypan blue dye-exclusion assays or by apoptosis assays.

T1

towards a rounded shape, as demonstrated by laser scanning confocal microscopy and scanning electron microscopy (Fig. 2, *G–J*). The morphology of the control HME-β-gal cells was also similarly affected, albeit to a lesser degree, by FTI treatment, as these cells became dissociated and flattened (Fig. 2, *A* and *B*).

Rho Protein Levels Increase as a Result of FTI Treatment. To determine the effect of FTI treatment on RhoC expression, we performed semiquantitative RT-PCR and Western blot analysis. As shown in Fig. 3*A*, RhoC mRNA expression increased in all of the cell lines on FTI treatment. A concordant increase in RhoC protein levels was also observed, as determined by Western blot analysis using a RhoC-specific antibody developed in our laboratory (33). The activity of RhoC was assessed using a GST-pulldown assay (34, 35). This assay utilizes a GST-fusion protein of a Rho-binding domain motif found in a variety of Rho-effector proteins. GTP-bound Rho is in its active state and can bind the Rho-binding domain (35). Using this assay, we found that in the SUM149 and HME-RhoC cell, the levels of GTP-bound RhoC were not affected by FTI treatment, which indicated that RhoC activation itself was unaffected by RhoB and RhoC accumulation. As expected from the mRNA and protein levels, activated RhoC was elevated in all of the FTI-treated cells, including the HME-β-gal control cells.

Because previous studies suggested a role for RhoB in reverting the malignant phenotype of Ras-transformed cells treated with FTI (42), we performed semiquantitative RT-PCR and Western blot analysis for RhoB. As shown in Fig. 3*B*, RhoB mRNA levels markedly increased 24 h after treatment with FTI L-744,832 in all of the cell lines tested. Furthermore, RhoB protein levels were also significantly increased.

These results support earlier observations that the accumulation of RhoB, likely RhoB-GG but not farnesylated RhoB, leads to a reversion of the malignant phenotype by FTI (30, 42, 43). The mechanism of FTI inhibition of the RhoC-induced phenotype appears to be independent of direct action of the FTI on geranylgeranylated RhoC. This is demonstrated by an accumulation of RhoC protein and no change in its activity.

Expression of RhoB-GG Recapitulates the FTI-mediated Effects on RhoC-overexpressing Breast Cells. In light of the previous experiments, we hypothesized that the accumulation of RhoB protein, specifically the RhoB-GG isoform that accumulates in FTI-treated cells, would be sufficient to revert the RhoC-induced phenotype. To test this hypothesis, we transiently transfected breast cells with a RhoB-GG construct and performed a focus formation and random motility assay. As controls, we also transfected cells with a geranylgeranyl-deficient mutant RhoB construct. Transfection efficiency was assessed by cotransfection with a *β-gal* reporter gene and found to be ~30–50% for the SUM149 and HME-RhoC cells. This assay could not be performed for the HME-β-gal controls since these cells already express the *β-gal* gene. However, RT-PCR using vector-specific primers demonstrated mRNA expression in all of the cell lines tested.

Table 1 shows the results of a focus formation assay for the transfectants. The SUM149 and HME-RhoC cells, tran-

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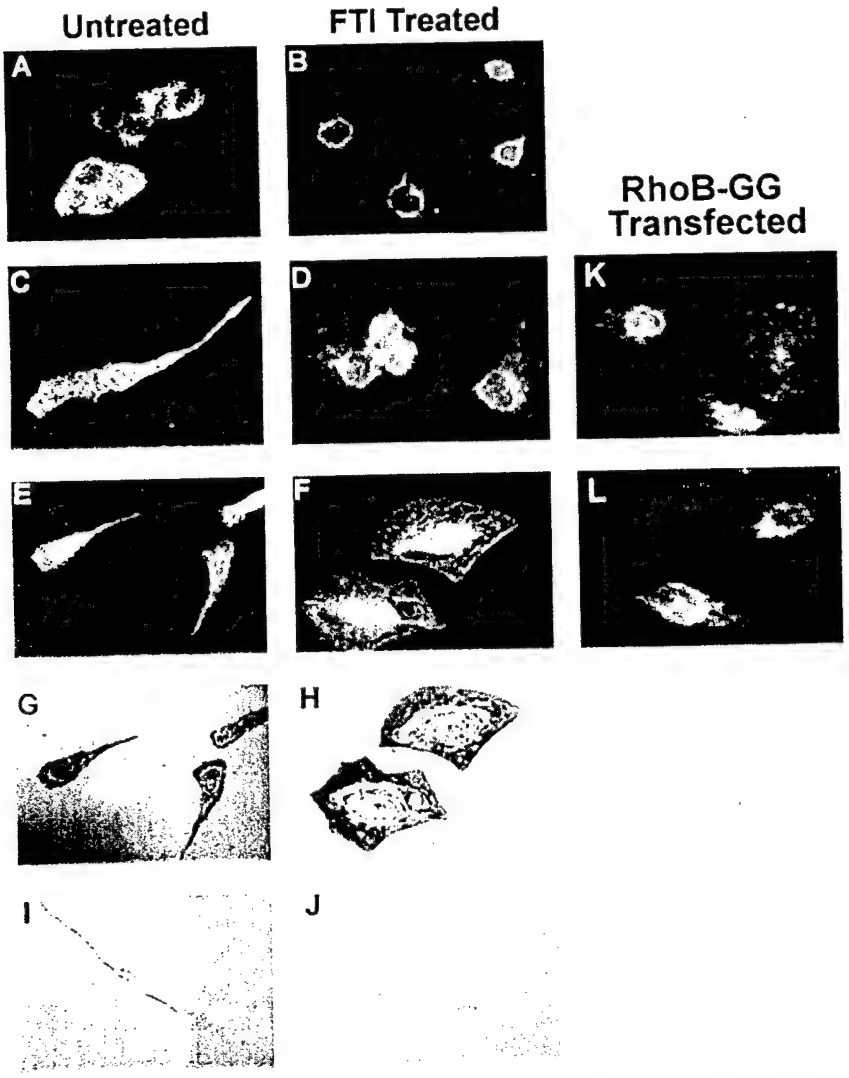
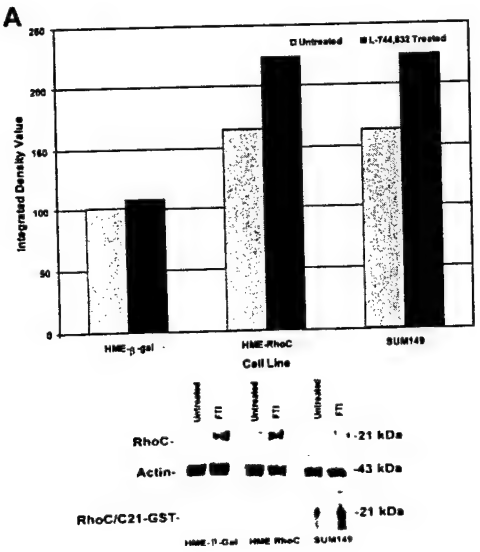


Fig. 2. Laser scanning confocal fluorescence microscopy (*A–F*) of untreated and L-744,832 treated cells after staining with a rhodamine-tagged phallotoxin that targets F-actin bundles. *A* and *B*, the HME-β-gal control cells untreated and FTI L-744,832-treated, respectively. *C* and *E* demonstrate a highly organized and polarized cytoskeleton in the SUM149 and HME-RhoC cells, respectively. *D* and *F* demonstrate dissociated actin bundles and decreased cellular polarity on FTI treatment. *G* and *H*, are laser scanning confocal microscopy images, and *I* and *J* are scanning electron microscopy images of untreated and FTI-treated HME-RhoC cells, respectively, showing the changes in cell morphology. *K* and *L* are RhoB-GG-transfected HME-RhoC and SUM149 cells, respectively.

siently transfected with RhoB-GG, formed significantly fewer foci than did the RhoB-GG-deficient mutant transfectants or the nontransfected cells. The morphology of the RhoB-GG-transfected cells were similar to their FTI-treated counterparts (Fig. 2, *K–L*). Similarly, the RhoB-GG transfectants were less motile when tested in the colloidal-gold assay (Fig. 4). Although statistical significance was not reached for the transfectants, the trends indicate that expression of F4



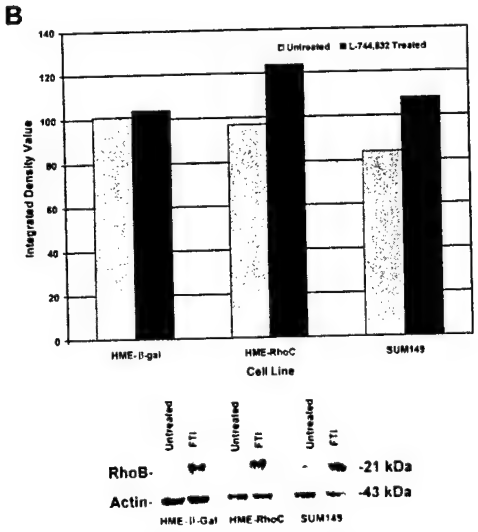


Fig. 3. Results of semiquantitative RT-PCR and Western blot analysis for RhoC GTPase (*A*) and RhoB GTPase (*B*). Densiotiometric comparison of the PCR products with a GAPDH internal standard demonstrated a modest increase in RhoC mRNA expression in all of the cell lines treated with FTI L-744,832 as compared with the untreated cell line. Similarly, an increase in RhoB mRNA expression was observed only for the HME-RhoC and SUM149 cell lines (*B*). A concurrent increase in RhoC protein levels was observed for the FTI-treated cell lines (*A*). This was accompanied by a slight increase in RhoC activity as determined by a C21-GST pulldown assay. As shown in *B*, RhoB protein levels increased in FTI-treated cells. *kDa*, *M*, in thousands.

Table 1 Mean focus formation and SD after transient transfection with RhoB-GG, a geranylgeranyl-deficient RhoB (RhoB-A3), and vector alone

The number of foci were assessed in triplicate 35-mm plates. The data presented are from a seeding density of 100 cells/plate. However, similar trends were seen when cells were seeded at 1000 or 500 cells/plate. Focus formation was dramatically reduced in the RhoC-overexpressing HME-RhoC and SUM149 cells by expression of geranylgeranylated RhoB, but not an unprenylated mutant (RhoB-A3). Although a trend was noted, statistical analysis by a Kruskal-Wallis test did not demonstrate a significant difference between the groups.

Cell line	Vector alone	RhoB-GG	RhoB-A3
HME-β-gal	48.5 ± 5.5	24.5 ± 1	36.5 ± 2.5
HME-RhoC	85 ± 3	23.5 ± 1	69 ± 6.5
SUM149	19.5 ± 1.5	5 ± 0.5	20.5 ± 1
MCF10AT c1	53.5 ± 0.5	25.5 ± 6	46 ± 1.5

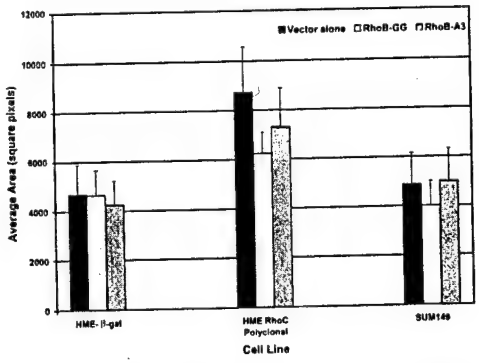


Fig. 4. Random motility assay of HME-β gal, HME-RhoC, and SUM149 cells transfected with either vector alone, RhoB-GG, or a geranylgeranyl mutant RhoB-A3. Although cell motility was not significantly altered, a trend was seen in the HME-RhoC and SUM149 cells transfected with RhoB-GG that resembled FTI treatment.

RhoB-GG leads to decreased motility in RhoC-overexpressing breast cells. These data provide evidence for a role for RhoB-GG as a mechanism for inhibiting or reverting the RhoC-induced phenotype in these cells.

Discussion

The observation that H-Ras was inactive and not localized to its specific membrane compartment after FTI treatment led to the idea that these inhibitors could be used therapeutically (44–48). Like Ras, the Rho GTPases are posttranslationally modified to locate them to their distinct cellular compartment, so that they can carry out their specific function (17–19). Each Rho protein contains a COOH-terminal CAAX domain that determines prenylation and polybasic residues in the hypervariable domain, upstream of the CAAX domain, which dictate proper membrane localization (49).

Membrane localization and trafficking of the Rho GTPases are a complex phenomenon. Rho proteins are, in their inactive state, localized in the cytosol, sequestered there by specific RhoGD (25). On activation, the GTP-bound protein is prenylated and transported to its specific membrane com-

partment. The type of prenylation is dependent on the Rho protein (50–53). RhoC GTPase is geranylgeranylated, whereas RhoB is both geranylgeranylated and farnesylated (17–19, 50, 51).

It has been demonstrated that *in vitro*, Rho GTPases can self-aggregate (54). Cdc42 and Rac2 homodimer formation has been implicated in the negative regulation of the activity of those proteins (55). RhoB and RhoC GTPase have been found to exist, not as homodimers, but as either monomeric or oligomeric complexes. In addition, RhoC has been shown to have an arginine finger motif COOH-terminal to the CAAX domain, which imparts self-activated GTPase regulatory function (54). Specifically, GTP-bound RhoC, when in complex with itself, can self-convert to RhoC-GDP. In contrast, RhoB does not contain this arginine domain and, therefore, does not have intrinsic GAP activity. It is yet unknown whether RhoB and RhoC can form heterodimers either *in vitro* or *in vivo*. Because FTI cannot directly block geranylgeranyl RhoC function, one possible explanation for FTI suppression of RhoC function is that accumulation of RhoB-GG leads to the oligomerization of RhoC, which leads to increased intrinsic GAP activity and GTPase deactivation.

The promise of FTIs as a potent therapeutic reagent has been supported by *in vivo* studies. Mammary tumors that develop in K-*Ras* transgenic mice can be growth inhibited by FTI treatment (56). In 1994, Lebowitz *et al.* (30) and Prendergast *et al.* (57–59) provided evidence that FTI suppression of Ras transformation was accomplished by interfering with Rho activity, because Rho was shown to be critical in Ras-induced transformation. Subsequent experiments demonstrated that a shift from farnesylated RhoB GTPase to RhoB-GG occurred on FTI treatment (30, 31, 60, 61). The shift in the specific forms of prenylated RhoB is accompanied by the accumulation and mislocalization of RhoB-GG, which is normally a short-lived protein (with a half-life of 2–4 h in cells; Refs. 30, 62). Our present data support these observations. In this study, we observed increased expression and accumulation of RhoB, presumably RhoB-GG, on treatment with FTI L-744,832. We also demonstrated that transient transfection of RhoC-overexpressing breast cells with RhoB-GG, recapitulated the effects of FTI treatment, inhibiting focus formation and random motility, whereas transfection with the GG-deficient mutant failed to mimic FTI effects.

In Ras-transformed cells, the effects of RhoB-GG may be attributable to a "gain-of-function" and relocalization of the protein (42, 43, 59, 63). Normally, RhoB GTPase is involved in vesicular and receptor trafficking (64). However, after FTI treatment, the inhibition of farnesylated RhoB and the accumulation of RhoB-GG, may lead to altered functions. The biosynthesis of geranylgeranyl PP_i is the next step after the synthesis of farnesyl PP_i in the acetyl-CoA pathway of cholesterol synthesis (reviewed by Cohen *et al.*; Ref. 48). Therefore, FTI treatment may provide more substrate for the geranylgeranyl PP_i synthase to produce geranylgeranyl PP_i and, ultimately, functionally geranylgeranylated Rho. Furthermore, several investigators suggest that a membrane receptor may exist that binds to the Rho prenyl-group, thereby helping to specifically localize it to a membrane compartment (51, 65–67). In this scenario, the accumulation of RhoB-GG may compete with RhoC, displacing it and preventing it from interacting with downstream effector molecules.

In our experiments, we demonstrate that on FTI-treatment, RhoC levels also increase; however, the ratio of RhoB:RhoC remains increased over pretreatment levels. Furthermore, we speculate that RhoC may be accumulating in the cytoplasm, or, if it is reaching the inner membrane, its effect on the cell is attenuated by RhoB-GG. These ideas have yet to be tested. As demonstrated by labeling the actin cytoskeleton with a rhodamine-labeled phallotoxin, the polarized actin bundles associated with the motile RhoC cells are nearly lost on FTI treatment. Although focal adhesions are visible in both treated and untreated cells, they are located exclusively around the outside edges of the FTI-treated cells. Both laser confocal and scanning electron microscopy demonstrate that, as observed previously (57), the cells have lost their polarity and are flattened.

In contrast with Ras-transformed cells, the growth rate of the RhoC-overexpressing cells was only slightly affected by FTI treatment. Cell viability was also unaffected. Again, this is in contrast to previous experiments, which demonstrated that a combination of FTI and LY294002 (an inhibitor of P13K) led to increased apoptosis in Ras-transformed cells (31) but not in RhoC-overexpressing cells.

Taken together, these data suggest that FTIs may prove a potent novel therapeutic agent against tumors that overexpress RhoC GTPase. This is the first report of FTI inhibition of the cancer phenotype induced specifically by overexpression of RhoC GTPase. The mechanism of FTI action in RhoC-overexpressing IBC and HME transfectants may be similar to that previously described for Ras-transformed cells, namely, that the effected is mediated by the accumulation of RhoB-GG. However, there are notable differences in how FTI treatment affects cell growth in Ras-transformed *versus* RhoC-overexpressing cells. In addition to IBC, it has been demonstrated that aggressive noninflammatory, metastatic breast cancers, advanced pancreatic cancer, and metastatic melanoma overexpress RhoC GTPase, and this event significantly contributes to their clinical behavior; therefore, FTI treatment may be effective against these aggressive cancers (33, 68, 69). Our data support testing whether FTI treatment is efficacious in these aggressive RhoC-driven malignancies.

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Detection of Epstein-Barr Virus in Rapidly Growing Fibroadenomas of the Breast in Immunosuppressed Hosts

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Fibroadenomas are the most common benign tumors of the female breast and are associated with a slight increase in the risk of subsequent breast cancer. Multiple fibroadenomas have been described in patients after renal transplantation and are thought to be secondary to drug-related growth stimulation. Epstein-Barr virus (EBV) has been detected in many neoplasms, including breast cancer. We set out to investigate whether EBV plays a role in the development of rapidly growing fibroadenomas in immunocompromised patients. We studied 19 fibroadenomas and one invasive ductal carcinoma that developed after organ transplantation or treatment for lupus erythematosus. As a control group we included 11 fibroadenomas from non-immunocompromised patients. DNA was amplified using polymerase chain reaction (PCR) of the EBV-encoded small RNA (EBER-2) DNA sequence. EBV latent membrane protein 1 (LMP-1) transcripts were amplified using reverse transcription (RT) PCR. Immunohistochemical (IHC) staining for LMP-1 protein was performed. A total of 9 out of 20 tumors (45%) were concordantly positive by PCR and IHC. IHC stained exclusively the epithelial cells. All the fibroadenomas in non-immunocompromised patients were negative for LMP-1 (Fisher's exact test $P = .0006$). These data suggest that EBV is associated with fibroadenomas in this immunosuppressed population and that the infection is specifically localized to epithelial cells. This is the first study suggesting a role for EBV in the pathogenesis of fibroadenomas.

KEY WORDS: Breast, Epstein-Barr virus, Fibroadenoma.

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Epstein-Barr virus (EBV) is a common human γ-herpes virus estimated to have infected about 95% of the adult population worldwide. Despite the widespread prevalence of EBV infection, EBV has been directly implicated in the development of only two malignancies observed in relatively restricted geographic areas: Burkitt's Lymphoma in East Africa and nasopharyngeal carcinoma in Southern China. It has also been closely associated to Hodgkin's Disease, thymic lymphoepithelioma, opportunistic B-cell lymphomas, pleural B-cell lymphomas or pyothorax associated B-cell lymphomas, opportunistic T-cell and NK-cell lymphomas in immunodeficiencies, primary nasal T-cell and NK-cell lymphomas, gastric carcinoma, and most recently, invasive breast carcinoma (1–9).

As the number of possible associations between γ-herpes viruses and human cancer continues to grow, the question of whether the individual's immune-response status is a predisposing factor for virally mediated neoplasms emerges. Patients with suppressed immune systems from HIV infection or post-organ transplant immunosuppressive drugs are important groups in whom to study viral carcinogenesis. Kaposi sarcoma, now known to be caused by human herpes virus-8 (HHV-8), is an AIDS-defining malignancy (10). Forty to 66% of transplant patients develop skin cancer within 20 years of the transplant procedure, presumably because cellular immunosuppression provides an opportunistic environment for cancer-associated vi-

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ruses (11). EBV-positive B-cell lymphomas are also present in about 20–30% of AIDS patients (12).

Fibroadenomas are benign breast tumors that have been associated with a slight increased risk of breast cancer (13, 14). The observation of rapidly growing fibroadenomas in immunosuppressed patients suggests a possible viral cofactor in the pathogenesis of benign breast neoplasms. In this study, we set out to test the hypothesis that EBV plays a role in the development of fibroadenomas in immunocompromised patients.

MATERIALS AND METHODS

Patient Sample Selection

Twenty patients with rapidly growing tumors of the breast were identified. The identification of rapidly growing was performed using mammographic evaluation. All the patients had masses growing faster than expected on mammograms from year to year. As controls, identified a group of 11 fibroadenomas in non-immunosuppressed patients. The formalin-fixed, paraffin-embedded breast tissues were retrieved from the Pathology files at the University of Michigan. The cases included 19 fibroadenomas and one invasive ductal carcinoma. Tissues were obtained by core biopsy (three cases) or by open biopsy (17 cases). All patients were either taking immunosuppressive drugs to prevent organ graft rejection or to control systemic lupus erythematosus. After selection, the tissues were stripped of identifiers referable to the patients.

DNA Extraction

Blocks were cut into sections and mounted on glass slides for analysis. Laser capture microdissection (LCM) technique was used for DNA extraction as previously described (15). Paraffin sections were deparaffinized, dehydrated, and stained with eosin. Individual tumor epithelial cells were microdissected off each section using a PixCell II LCM system (Arcturus, Mountain View, CA). Laser pulses were used to fuse individual cells onto the surface of transfer film/microcentrifuge tube caps. Digestion buffer (0.04% Proteinase K, 10 mM Tris-HCL (pH 8.0), 1 mM EDTA, and 1% Tween-20) was added to the tubes. The capped tubes were inverted and incubated overnight at 37° C. Samples were boiled for 5 minutes at 95° C and, if not used immediately, stored at −20° C. As positive control we used DNA from a known EBV-positive testicular lymphoma.

PCR Amplification

Primers for the EBV-encoded small RNA (EBER-2) DNA sequence were synthesized as previously described (5'-CCCTAGTGGTTTCGGACACA-3' sense primer and 5'-ACTTGCAAATGCTCTAGGCG-3' anti-sense primer) (2). PCR reagents and reaction protocols were tested using pilot runs with DNA extracted from an EBV-positive testicular lymphoma. Single-round PCR of our LCM-extracted tumor DNA (15 μL) were then performed using the following conditions: denaturation at 95° C (5 minutes), followed by 40 cycles of denaturation at 95° C (30 seconds), annealing at 58° C (1 minute), and extension at 72° C (2 minutes). The cycle was followed by a 7 minute extension at 72° C.

To determine whether the extracted DNA was technically suitable for PCR amplification, a region of the beta-tubulin gene sequence was concurrently amplified (5'-AAATTTTAACCATGAGGGAAATC-3' sense primer and 5'-GTGGGGTCGATGCCATGT-3' anti-sense primer). Single-round PCR was performed as follows: denaturation at 95° C (5 minutes), followed by 35 cycles of denaturation at 95° C (1 minute), annealing at 58° C (1 minute), and extension at 72° C (2 minutes), ending with a 7 minute extension at 72° C. Both EBER-2 and beta-tubulin PCR products were separated on a 2.5% agarose gel by electrophoresis and visualized with ethidium bromide under UV light.

RT-PCR Amplification

Tissue was scraped off paraffin embedded sections and onto siliconized 2.0 ml microcentrifuge tubes (Life Science Products, Denver, CO) for RNA extraction. Two slides were scraped for each sample, deparaffinized in xylene and washed in ethanol. After centrifugation at 20,800 RCF for 10 minutes, Trizol RNA isolation reagent (Life Technologies, Rockville, MD) was added to the dried pellets. Tubes were then incubated at 65° C for 10 minutes. Sample tubes were placed on ice and *E. coli* tRNA (Roche, Basel, Switzerland) was added. The samples were mixed vigorously with chloroform and incubated for 3 minutes. The microcentrifuge tubes were centrifuged again at 4° C for 15 minutes (15,300 RCF). The clear aqueous phase was transferred to an RNase-free microcentrifuge tube. RNA was then precipitated in isopropanol and centrifuged for 4° C for 10 minutes. The pellet was washed in 75% ethanol, 100% ethanol, and resuspended in Diethyl Pyrocarbonate-treated distilled and deionized water (DEPC ddH_20). Samples were stored at −80° C. RNA was also extracted from an EBV-positive testicular lymphoma to serve as a positive control.

A one-tube reverse-transcription/cDNA amplification method followed by a nested cDNA PCR amplification was used. Nested primers (5'-ATCTTCGGGTGCTTACTTG-3' external sense, 5'-AAGGCCAAAAGCTGCCAGATGGTGGC-3' external anti-sense, 5'-CATTGTTCCTTGGAATTGTGCTGT-

TC-3' internal sense, and a 5'-ACCAAGTCGC-CAGAGCATC-3' internal anti-sense) were designed to span an intron in the EBV genome, so amplification of cDNA would yield a different size band than amplification of genomic DNA.

An initial PCR reaction mix of 0.2 mM DNTPs, 1X PCR buffer (Promega, Madison, WI), 1.4 mM $MgCl_2$, 10 ng/μl sense and anti-sense LMP-1 primers, and DEPC ddH_20 was made. Before each reaction, 2.5 U/rxn Taq polymerase (Promega), 12.5 U/rxn Reverse Transcriptase AMV (Roche), 40 U/rxn RNase Inhibitor (Roche) were combined with the initial PCR reaction mix. This final reaction mix was then aliquoted to individual reaction tubes and sample RNA was added. The solution was then cycled through the following steps: a reverse transcription at 42° C (30 minutes), denaturation at 95° C (4 minutes), and 35 cycles of 95° C (1 minute), 57° C (2 minutes), 72° C (2min), followed by a final extension of 72° C (7 minutes). After completion of the RT-PCR reaction, 5μl of cDNA product was used in a nested PCR reaction with internal primers. They were then cycled through the same program as previously described for the tubulin PCR reactions. Products were separated on a 2.5% agarose gel and visualized with ethidium bromide under UV light.

Immunohistochemistry

A DAKO LSAB2 HRP (DAB) staining kit was used for LMP-1 immunohistochemistry (IHC). Samples were analyzed with primary mouse monoclonal antibodies recognizing four distinct LMP-1 epitopes (DAKO) according to the manufacturer's instructions. Paraffin embedded sections were first deparaffinized twice in xylene for 5 minutes, then rehydrated for 3 minutes in absolute ethanol twice and 95% ethanol twice. Sections were pretreated with pronase (DAKO) to break down formalin-crosslinking. The samples were counterstained in Mayer's Haemtatoxylin (DAKO) for 5 minutes and dipped 10 times into 37 mM ammonia. Slides were then treated with aqueous crystal mounting medium (Biomeda) and coverslipped with Permount (Fisher). Sections from a paraffin-embedded EBV-positive hu-PBL-SCID lymphoma were stained as positive controls. Appropriate negative controls were used in each run.

RESULTS

Clinical Findings and Histologic Study

Table 1 summarizes the clinicopathologic information. All patients were women, ages 21 to 65 at the time of diagnosis. Examination of hematoxylin and eosin stained sections revealed that 19 of the 20 tumors were fibroadenomas with classic histologic features of elongated and attenuated ducts, compressed by a fibrous proliferating stroma (Fig. 2A and B). The single case of carcinoma was an invasive ductal carcinoma with associated ductal carcinoma *in situ*. None of the cases had prominent lymphocytic infiltrates.

Detection of EBV-Specific Sequences and Protein

Thirteen of the 18 (72%) fibroadenomas were positive for EBER-2 sequence (Table 1, Fig. 1). Amplification of the tubulin gene confirmed that the DNA was suitable for PCR amplification. EBER-2 DNA sequence was successfully amplified from the extracted DNA of a paraffin-embedded EBV-positive testicular lymphoma. The water control (in which no sample DNA was added) did not yield a

TABLE 1. Clinicopathologic Information

Case	Clinical History	Pathologic Diagnosis	Age	EBER-2 PCR	LMP-1 IHC
1	Kidney transplant	Fibroadenoma	43	NA	Pos
2	Heart transplant	Fibroadenoma	56	Pos	Pos
3	Kidney transplant	Fibroadenoma	34	Pos	Pos
4	Lupus	Fibroadenoma	44	Pos	Pos
5	Heart transplant	Fibroadenoma	49	Pos	Neg
6	Kidney transplant	Fibroadenoma	42	Pos	Pos
7	Kidney transplant	Fibroadenoma	45	Pos	Neg
8	Heart transplant	Fibroadenoma	43	Pos	Pos
9	Liver transplant	Fibroadenoma	35	Pos	Pos
10	Lupus	Fibroadenoma	25	Pos	Pos
11	Kidney transplant	Fibroadenoma	21	Neg	Pos
12	Kidney transplant	Fibroadenoma	26	Pos	Pos
13	Kidney transplant	Fibroadenoma	25	Neg	Pos
14	Heart transplant	Fibroadenoma	53	Pos	Neg
15	Heart transplant	Fibroadenoma	53	Pos	Pos
16	Liver transplant	Invasive ductal carcinoma	65	NA	Neg
17	Kidney transplant	Fibroadenoma	44	Neg	Neg
18	Kidney transplant	Fibroadenoma	44	Pos	Neg
19	Kidney transplant	Fibroadenoma	44	Neg	Neg
20	Kidney transplant	Fibroadenoma	44	Neg	Neg

product, indicating the reaction was free of contamination, an important concern in this experiment.

Fourteen tumors were analyzed by nested RT-PCR for LMP-1 RNA. RNA extracted from an EBV-positive testicular lymphoma was amplified simultaneously with the samples, controlling for the RNA extraction technique, PCR conditions, and reagents. The primers were designed to target an intron sequence to yield an expected product of 258-bp. In contrast, amplification of LMP-1 DNA contamination was expected to produce a 333-bp product, and thus, easily recognized. Although the testicular lymphoma consistently displayed the expected RNA band, none of the breast fibroadenoma samples showed evidence of LMP-1 RNA, likely due to minute yields of this RNA species.

All 20 tumors from immunocompromised patients and 11 tumors from non-immunocompromised patients were immunohistochemically stained with antibodies for LMP-1. Results were scored independently by two investigators. The hu-PBL-SCID lymphoma control consistently displayed strong staining of the EBV-transformed human lymphocytes. As expected, surrounding mouse tissue did not stain positively for LMP-1. Twelve fibroadenomas from immunocompromised patients (60%) expressed LMP-1 protein in the cytoplasm of the epithelial cells (Fig. 2). The single case of invasive ductal carcinoma was negative for LMP-1. All fibroadenomas from non-immunocompromised patients were negative (Fisher's exact test, $P = .0006$). Adipocytes, normal breast lobules and stromal cells were negative in all cases. Table 1 summarizes the results of EBER-2 PCR, LMP-1 RT-PCR, and LMP-1 immunohistochemistry.

DISCUSSION

Although the correlation between EBV and lymphoproliferative disorders is strongest, EBV has also been associated with several cancers of epithelial origin, such as nasopharyngeal carcinoma and gastric carcinoma (1, 2, 9). In addition, EBV has long been suspected to play a role in human breast carcinogenesis. The presence of a mouse mammary tumor virus in rodents and published reports of HIV-positive rapidly growing breast cancers suggest a possible viral etiology for some breast cancers (10, 16). One study reported multiple and bilateral fibroadenomas in immunosuppressed patients receiving cyclosporin A (17). Isolated studies have investigated the EBV/breast cancer association with contradictory results (2, 18, 19).

This is the first study to investigate the role of EBV in rapidly growing breast tumors in immunocompromised patients. We detected the EBV genome, through PCR amplification of EBER-2 DNA, in 13 of 18 (72%) tumors (Fig. 1), and LMP-1 protein in 12 of 20 (60%) (Fig. 2) of the tumors studied. The fact that none of the tumors had lymphocytic infiltration minimizes the possibility that the positive EBER-2 results were due to contaminating lymphocytes. Nine tumors (45%) were concordantly positive for both, EBER-2 DNA and LMP-1 protein. None of the fibroadenomas from non-immunosuppressed patients expressed LMP-1 protein (Fisher's exact test $P = .0006$). Therefore, we conservatively estimate that EBV is associated with the development of fibroadenomas in immunocompromised patients in 45% of the cases but not in non-immunocompromised patients. We did not, however, detect LMP-1 RNA in our samples using nested RT-PCR. This was likely due to the low sensitivity of our assay and the paucity of RNA material extracted from tumor sections. Although our protocol was developed from several published reports of RNA isolation from paraffin embedded tissues, we were unsuccessful in determining whether our extracted RNA was technically suitable for PCR and thus, we were unable to replicate in our samples previously published results (20). The projected yield of RNA for each sample was so marginal that co-amplification of tubulin RNA would have jeopardized the RT-PCR of LMP-1 RNA. It is also possible that levels of LMP-1 message are too low in fibroadenomas to be detected through RT-PCT, yet are sufficient to translate proteins and participate in the pathogenesis of fibroadenomas.

We have established for the first time an association between EBV infection and fibroadenomas of the breast in immunosuppressed patients. The mechanism by which EBV may be implicated in breast tumorigenesis has not been elucidated. It appears from the epidemiologic literature that EBV infection alone is not sufficient for breast tumor development in humans. This assertion is consistent with the existence of EBV-positive benign breast tumors. In this study, we localized EBV to epithelial cells within tumor samples. It is possible, that EBV converts the epithelial cells to a preneo-

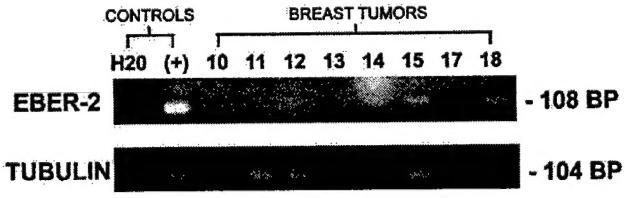

FIGURE 1. Detection of Epstein-Barr virus genome by PCR amplification of the 108-bp EBV-encoded small RNAs (EBER-2) fragment. A 104-bp b-tubulin sequence was amplified to verify the presence of genomic DNA in each sample extraction. H_20 = water control without DNA and (+) = EBV-positive control (testicular lymphoma). Samples 10, 12, 14, 15, and 18 = positive tumors; 11, 13, and 17 = negative tumors.

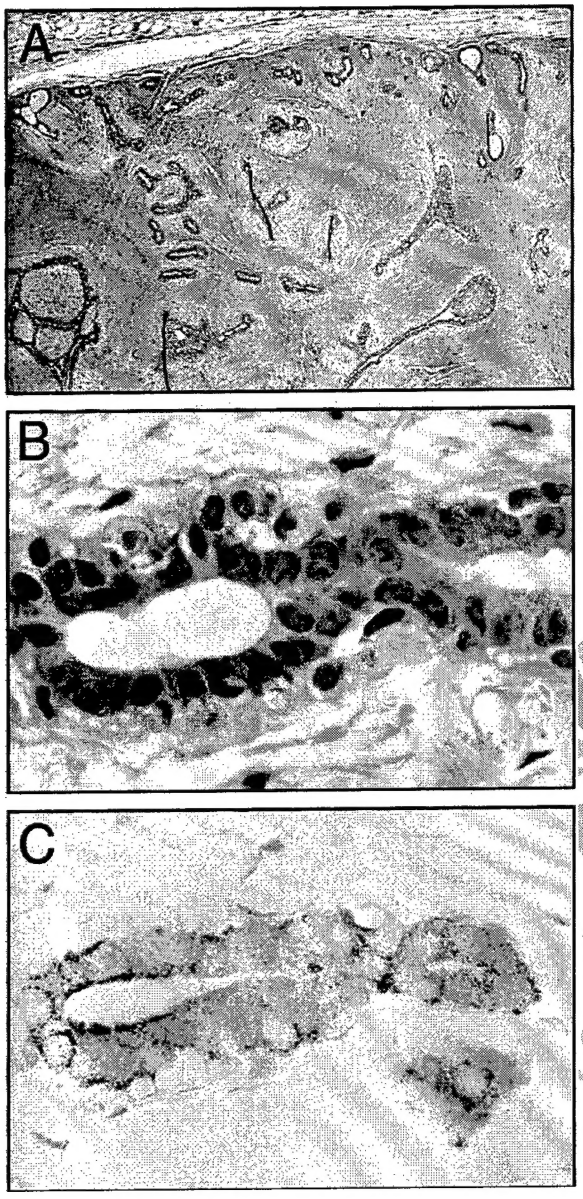

FIGURE 2. **A**, Low power (100×) photograph of a fibroadenoma in an immunocompromised patients. There are no distinctive histologic features; **B**, High power (400×) picture of a duct in this fibroadenoma. **C**, Same fibroadenoma with cytoplasmic immunostaining for LMP-1 (400×). Note the positive membrane associate LMP-1 staining in the ductal epithelial cells and the negatively stained stromal fibroblasts.

plastic state through increased proliferation of epithelial cells in a similar way that EBV induces immortalization of B-lymphocytes (1, 21).

Although we did not set out specifically to do so, our results may help reconcile some of the conflicting results from previous studies that investigated EBV in breast tumors. Among our samples, a significant proportion (72 %) were positive by at least one technique (EBER-2 PCR). In the Bonnet (2) and Labreque (18) studies, in which they demonstrated the presence of EBV genome using PCR, only a handful of samples were analyzed by another EBV detection technique such as IHC. In the negative studies of Chu *et al.* (22, 23) and Glasser *et al.* (19), EBER-2 PCR amplification was not performed. Our results show that different EBV detection techniques often display seemingly contradictory results, possibly because their sensitivity depends on sample-specific preparation at the time of fixation, or subsequent alterations of the specimens over time. The difference between previous studies showing positive and negative associations between EBV and breast cancer may be due to choice and number of detection modalities. Therefore, although EBV-mediated breast cancers may have different patterns of EBV-specific gene expression than EBV-mediated fibroadenomas, our data suggest that future studies of this kind should involve at least two EBV screening approaches to confirm potential associations.

In conclusion, we have demonstrated that EBV is specifically present in the epithelial cells of a well-defined subset of breast tumors. This is the first study to show the association between EBV and benign breast neoplasms. Furthermore, the localization of EBV exclusively in the tumor epithelial cells suggests that EBV may play a causative role in the development of breast fibroadenomas in immunosuppressed patients.

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